The influence of metal-on-metal wear on infection in hip arthroplasty

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Chapter 1

Introduction and aim of this thesis

Worldwide, an increasing number of biomaterial implantations are performed for the restoration of human function to ensure a high quality of life during aging. Of these operations, hip arthroplasty is considered one of the most successful. The success of this frequent procedure has led to large patient cohorts. Notwithstanding a vast improvement in surgical protocols, the complication of a biomaterial-associated infection still occurs in around 1% of the patients receiving a hip or knee replacement (1). Although 1% may seem like an acceptable risk, deep infections and the accompanying high morbidity remain a significant problem in modern medicine due to the large numbers of implant operations (9).

Two decades ago, metal-on-metal (MOM) bearings were reintroduced on the market. These "new" implants were initially praised because of their low wear rates compared to the metal-on-polyethylene (MOP) bearings. MOM implants have since been used frequently, primarily in the United Kingdom, Australia and the United States (2;3;7). However, MOM bearing surfaces are currently becoming under scrutiny of the scientific community (4), partly because of potential side-effects of their wear particles. Research institutions have been focusing on the influences of metal particle debris in the human body, but due to its recent introduction and the shortage of long term studies (6;8), the clinical outcome is still unclear. Moreover, if and how MOM implants would influence infection rates compared to conventional MOP bearings remains uncertain (5;8). The host defense mechanism is compromised in the presence of a foreign material: the mere presence of a foreign body itself reduces the minimum number of bacteria required to cause infection (10). However, the influence of wear products from modern orthopaedic bearing materials on infection is not yet established.

The aim of the work described in this thesis is to assess the influence of MOM bearing wear on infection risk. Specifically, this involves the effects of Co-Cr ions and particulate Co-Cr debris on bacterial growth. The majority of the studies included in this thesis are *in vitro* studies, while two final chapters deal with an animal study on the effects of different particulate debris on infection and, lastly, a national joint registry study comparing infection risk in MOM and MOP patients.

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Chapter 2

Metal-on-metal wear effects on the host's immune system and infection in hip arthroplasty

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Abstract

Background

Joint reconstructions with metal-on-metal (MOM) bearings have gained popularity in the last decades in young and active patients. However, possible effects of MOM wear debris and its corrosion products are controversial. Alongside potential disadvantages such as toxicity, influences of metal particles and metal ions on infection risk are unclear.

Methods

We reviewed available literature on the influence of degradation products of MOM bearings in hip arthroplasties on infection risk.

Results

Wear products were found to influence the risk of infection by hampering the immune system, inhibiting or accelerating bacterial growth and by a possible antibiotic resistance and heavy metal co-selection mechanism.

Conclusion

Whether the combined effects of MOM wear products predispose MOM bearings as being less or more prone to infection, needs to be investigated in the near future.

Introduction

Many young patients with painful coxarthrosis want to return to a high level of activity and seek an implant that provides durability. Low wear rates of metal-on-metal (MOM) bearings have led to a resurgence in the use of MOM bearings (27;79;95;106;110). Thirty-five percent of all prostheses in the United States in 2006 (7) and 16% of all prostheses implanted in Australia from 1999 to 2007 contained MOM bearings (40).

Metal alloys used in MOM bearings degrade by wear, corrosion, or by a combination of them (49;118). Consequently, MOM bearings produce nanometer to submicrometer-sized metal particles (19;30). The high number of these very small particles presents a large cumulative surface area for corrosion. The biological influences of these particles and its corrosion products in the human body are for the most part unclear. Since the renewed interest in MOM bearings, extensive research has been done to determine the consequences of local and systemic exposure to wear particles and accompanying biologically active corrosion products (2). It is well known that metal debris can induce pathological changes such as the release of inflammatory cytokines from macrophages, histiocytosis, fibrosis, or necrosis (5;15;16;39). Metal debris is also thought to be associated with hypersensitivity and osteolysis (20;38;41;42;47). However, literature is scarce on the bacteriological effects of these degradation products (3;45). It is therefore unclear if the risk of infection is influenced.

The Australian and the New Zealand Joint Registry have demonstrated that between 9-15% of all total hip arthroplasty (THA) revisions are carried out because of infections related to the primary prosthesis (40;85). In case of infection, bacteria adapt a biofilm mode of growth on the surface of the prosthesis, increasing antibiotic resistance of biofilms which result in major treatment difficulties (102). Removal and replacement of an infected implant is usually required to eliminate the infection (8;107). Recent research efforts suggested that particulate debris, of any composition, promotes bacterial growth by providing a scaffold for bacterial adhesion and biofilm growth (3). On the other hand, high concentrations of metal ions have been shown to possess bacteriostatic properties (45).

Considering the paucity in publications on MOM particle influences on infection, we here review the literature on influences of MOM wear particles and its corrosion products on the risk of infection.

MOM bearings

History

First-generation MOM hip bearings encompass prostheses developed in the 1960s, such as the McKee-Farrar-, the Ring-, the Stanmore- and the Sivash-prostheses (66;84;89;98). Implants from this era survived for more than 25 years because of low wear rates and minimal osteolysis (2). An analysis of 253 Ring MOM hip arthroplasties revealed a cumulative survival rate of 60% after 21 years (12). The McKee-Farrar prosthesis performed equally well compared to the Ring arthroplasty up to 26 years after initial implantation (91). However, alongside these encouraging durability results, first-generation MOM studies also demonstrated metal wear debris in tissues adjacent to the implants, particularly in prostheses with loose components or impingement (46). Furthermore, early MOM designs turned out to cause frequent early cup loosening (91).

First-generation MOM articulations were commonly used until the mid 1970s. Most were abandoned in favor of metal-on-polyethylene (MOP) articulation. The main reason for this change was the introduction of the Charnley low-friction arthroplasty (23), which is still one of the most extensively documented hip prosthesis in the literature (17;18;117). Long-term results of first-generation MOM implants had boosted their popularity and lead to development of second-generation MOM implants in the early 1980s. In addition, polyethylene wear from MOP implants had then been hypothesized to cause osteolysis around the implant (77;116), which stimulated renewed interest in alternative bearings lacking a MOP interface, like the second-generation MOM bearings (11).

Second-generation MOM implants possess an improved bearing interface and are comprised of alloys with an increased metal hardness. Therefore, newly produced bearings have substantially lower wear rates than highly cross-linked polyethylene (36). On the whole, volumetric wear is decreased 20 to 100-fold compared to MOP implants (95), suggesting second-generation MOM prostheses may considerably reduce osteolysis (94). Although mid- and long-term clinical

results of MOM bearings appeared to have demonstrated excellent durability, recent studies show that MOM bearing systems are not immune against osteolysis (55).

Alloys

For implant alloys worldwide, two nomenclatures are practiced parallel to each other (Table I). First of all, ASTM-standards with the capital "F" (medical devices) are practiced mainly in the USA (44). Secondly, ISO-standards are accepted in the rest of the world. The two approved cobalt-chromium (Co-Cr) alloys contain almost similar amounts of alloying elements. However, information on the exact content of certain elements such as nickel (Ni) and iron (Fe) is unknown as these standards only report maximum dosages.

Table I. Required chemical composition and mechanical properties of CoCr alloy concerning ASTM F75 and ISO 5832-4

	ASTM F75	ISO 5832-4
Rm[a] (MPa)	655	665
Rp 0.2%[b] (MPa)	450	450
Cobalt, Co	balance to 100%	balance to 100%
Chromium, Cr	27-30%	27–30%
Molybdenum, Mo	5.0-7.0%	4.5-7.0%
Nickel, Ni	<0.5%	<1%
Iron, Fe	<0.75%	<1%
Silicon, Si	<1%	<1%
Manganese, Mn	<1%	<1%
Carbon, C	<0.35%	<0.35%

[a]Tensile strength is the stress at which a material breaks or permanently deforms.
[b]Yield strength is the stress at which a material begins to deform plastically.

Wear products

Wear

Wear of bearings can result from scratching and pounding of the surfaces and, eventually, in erosion of the material. Wear and corrosion are probably the major cause of metal release into tissues of MOM patients and this poses a major concern regarding the use of MOM articulating devices. Linear wear rates range from 5 to

25 µm/year and are dependent on a multitude of factors such as the type of implant and positioning (76;93;114) (Table II).

Table II. Wear rates of MOM bearing couples defined in different units

Type of wear	Wear rate	Method	Ref.
Linear wear rate of femoral heads per year	7.6 µm (range 2.9-13) to 250 µm (range 50-810)	Explanted implant(s) Radiograph analysis	(83) (100)
First year	25 µm	Explanted implant(s)	(94)
> 3 year	5 µm	Explanted implant(s)	(94)
Volumetric wear rate of femoral heads per year	2.0 mm^3 (range 0.55-3.7)	Explanted implant(s)	(83)
	5.0 mm^3 (range 0.22-22)	Explanted implant(s)	(112)
Mass wear rate per year	17 mg (range 4.6-31)	Explanted implant(s)	(83)
Number of particles per unit volume of wear (mm^3)	2.7×10^{12} to 1.5×10^{13}	Pin-on-plate	(101)
Number of particles per 10^6 cycles	$4 \times 10^{12} - 6 \times 10^{13}$	Pin-on-plate	(101)
Number of particles per year	$6.7 \times 10^{12} - 2.5 \times 10^{14}$	Explanted implant(s)	(30)

Note: Retrieval study data was obtained from patients undergoing revision of THAs with MOM bearing couples. Radiograph wear analysis was performed with digitized anteroposterior (AP) radiographs with a computerized method.

Metal particles

Currently, tribological research is being conducted towards the exact process of particle generation. Recent tribological investigations revealed that a nano-crystalline layer of 250 to 400 nm thick is formed on the MOM implant surfaces, containing amongst others proteins from the interfacial medium (81). Cracking of this nano-crystalline layer due to surface fatigue has been suggested to be the main mechanism of wear particle generation (13). Abrasive particles of MOM prostheses can cause local damage, resulting in an accelerated release of metal particles and ions (119).

Germain et al. (2003) emphasized that nature, size, and amount of particles are important determinants in biological effects of wear debris on cells *in vitro*. The reaction of the body is dependent on the particle characteristics (Table III). Size analysis of particles isolated from failed arthroplasties revealed a mean size of 660 nm for polyethylene particles in MOP patients (68) and a size range from 51 to 116 nm for MOM debris (29).

Metal ions

The first report on visible corrosion of an orthopedic Vitallium implant (60% Co and 20% Cr) was published by Weightman et al. in 1969. Before this clinical finding, it

was generally accepted that Vitallium alloys provided adequate corrosion resistance (90). However, generation of metal ions is also evident in modern, more corrosion resistant, MOM alloys.

Various mechanisms of corrosion can cause the release of metal ions. One is fretting corrosion due to the movement of articulating surfaces causing damage to one or both surfaces. Disruption of the passive oxide layer causes direct contact with the metal surface promoting fretting corrosion (Figure 1), which can be further enhanced by the presence of adhering microorganisms (70).

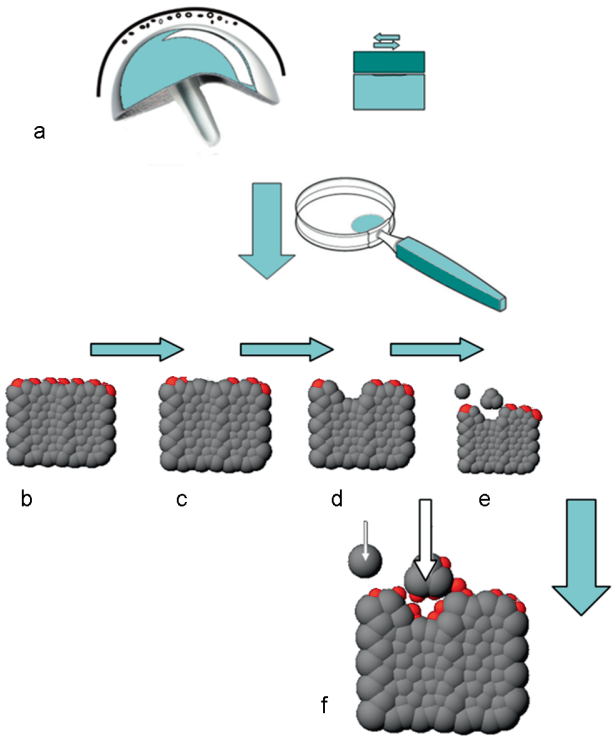

Figure 1. Schematic drawing illustrating:

a. Generation of wear particles
b. A metal alloy (grey scaffold) with an oxidated surface film (red molecules) on the upper surface.
c. Damaging of the passive surface film (e.g. by scratching or pounding).
d. Corrosion occuring due to the lack of a protective layer.
e. Soluble compounds and wear particle liberation.
f. Repassivation of the surfaces including wear particles (arrows).

Metal ions of different valencies are released from Co-Cr alloys and their effects vary with the type of oxide compound they form (108) (Table IV). Co^{+2} and Cr^{+3} ions dominate under physiological conditions because these ions are the most stable at neutral pH. However, no stable Co-oxide exists and thus formation of soluble Co-ions instead of solid Co-oxides is favored. On the other hand, Cr^{+3} oxides are stable in physiological conditions. *In vitro* models show that toxic effects of Co-Cr are probably due to Co^{+2} ions (37;82).

Table III. Size and morphology of wear particles generated by a hip simulator or derived from tissue samples

Size and morphology	Particles generation and type of prosthesis	Method	Ref.
80 ± 40 nm, round	Hip simulator with bearing ASTM F 799 and F1357 Co-28Cr-6Mo	TEM	(21)
50-90 nm, oval or needle	Hip simulator with bearing ASTM F 799 and F1357 Co-28Cr-6Mo	SEM	(101)
25-36 nm, round	Hip simulator with bearing ASTM F 799 and F1357 Co-28Cr-6Mo	TEM	(35)
< 50 nm (range 6-834 nm), oval or round	Periprosthetic tissue samples of 2 McKeeFarrar and one McMinn prosthesis	TEM	(30)
< 50 nm, irregular	Periprosthetic tissue samples of 640 Sikomet SM21	SEM	(10)
40-120 nm, needle <90 nm, round	Periprosthetic tissue samples of one Bicon plus Ti shell with polyethylene liner, Sikomet SM21 head and SL-Plus stem	TEM, SEM & XPS	(67)

TEM: Transmission electron microscopy
SEM: Scanning electron microscopy
XPS: X-ray photoelectron spectroscopy

Table IV. Oxidation states found in compounds of the Co-Cr alloy elements

Cobalt	Chromium	Manganese	Iron	Nickel	Silicon	Molybdenum
-1	-2	-3	-2	-1	-4[a]	-2
+1	-1	-2	-1	+1	-3	-1
+2[a]	+1	-1	+1	+2[a]	-2	+1
+3[a]	+2	+1	+2[a]	+3	-1	+2
+4	+3[a]	+2[a]	+3[a]	+4	+1	+3
+5	+4	+3	+4		+2	+4[a]
	+5	+4[a]	+5		+3	+5
	+6[a]	+5	+6		+4[a]	+6[a]
		+6				
		+7[a]				

[a] represents more common oxidation states.

Local concentrations

Metal ions may spread throughout the body. Ion levels have been measured in whole blood, serum, erythrocytes and various solid tissues (24;58;87). Serum Co levels are the most frequently reported metal ion concentrations and were found to be 5 to 6-fold greater in patients after MOM-implantation than pre-operatively (58).

Two well-received consensus papers describe the need to measure metal ion concentrations in joint fluids of MOM patients (1;65), in addition to measuring metal concentrations in serum (87;106). However, most research into metal ion concentrations does not report the regional or local dissemination, as synovial biopsies are undesirable in otherwise healthy MOM patients (87).

Reliable information about the exact local concentrations of Co–Cr around prostheses is thus scarce (Table V). Concentrations of metal ions are found with a range of 6 to 6,000,000 µg/L Co. This difference is related to alignment and type of the prostheses (76;93;114), but also to improving detection methods (31).

Table V. Maximum local tissue Co-Cr ion levels in patients with a MOM implant.

Sample	Prosthesis	Cobalt (µg/L)	Chromium (µg/L)	Method	Ref.
Capsule	Cemented and loose	26,000	88,000	NAA	(33)
Synovial fluid Capsule	Cemented and loose	250 22,000		NAA	(51)
Femoral neck Acetabulum Lining from femoral stem Adjacent to articular surfaces Acetabular pelvic lining	Cemented	50,000 170,000 130,000 70,000 200,000	170,000 170,000 1,300,000 70,000 200,000	SES	(99)
Capsule	Loose	6,000,000	1,500,000	AAS/ NAA	(80)
Synovial fluid Capsule Granuloma Tissue (mid femur)	Cemented and loose	13,000 63,000 193,000 6900	63,000 327,000 323,000 5500	GSGSD	(28)
Synovial fluid	Cemented and loose	155	358	AAS	(25)
Synovial fluid Capsule Fibrous membrane Synovial fluid Capsule Fibrous membrane	Cemented (well fixed and loose) Cementless (well fixed and loose)	199 3971 2451 1015 1272 3812	347 1465 1634 617 6219 20,609	GFAAS	(31)
Synovial fluid Synovial fluid	Cemented and well fixed Cemented and loose	6 152	16 238	GFAAS	(9)

NAA: Neutron activation analysis
SES: Spark emission spectroscopy
AAS: Atomic absorption spectroscopy
GFAAS: Graphite furnace atomic absorption spectrophotometry
GSGSD: Gamma-Spectroscopy with Ge-Semiconductor Detector

Table VI. Maximum levels of Co-Cr ions as measured in hip simulator lubricant, categorized by MOM implant head size and amount of cycles.

Head size	Cobalt (µg/l)	Chromium (µg/L)	Method	Cycles	Ref.
55 mm 39 mm	± 18,000 ± 12,000	± 6000 ± 4000	ICP-MS	0.13 Mc	(59)
55 mm 39 mm	10,915 9066	3675 3302	ICP-MS	0.13 Mc	(60)
36 mm 28 mm	± 6,800,000 ± 12,000,000	± 2,800,000 ± 8,000,000	ICP-MS	4 Mc	(113)

ICP-MS: Inductively coupled plasma mass spectroscopy
Mc: Million cycles

Wear rates reported in recent hip simulator studies turn out to be closely correlated to Co ion levels (113), but measuring ion levels in the lubricant of hip simulators resembles only certain aspects of the clinical situation. There is no free exchange between blood and synovial fluid (31) and, therefore, wear products can accumulate in simulator systems resulting in higher Co-Cr levels than would occur clinically (Table VI).

Influence of MOM wear particles and corrosion products on the immune system

Orthopaedic metals and their corrosion products modulate the activities of the immune system by influencing immunocompetent organs and cells by a variety of immuno-stimulatory or immuno-suppressive mechanisms. Metal particles and ions spread throughout the whole body via lymph and blood have, for instance, been identified within macrophages in the liver and spleen (104;105). The long-term effects of the distant spread and accumulation of wear particles in the liver and spleen are unknown, but indicate that the immune system may be hampered. In this context, it should be emphasized that the mere presence of a foreign body itself already reduces the minimum number of bacteria required to cause infection (122).

Another important issue is the hypothetical carcinogenesis due to MOM implants and its accompanying occupation of the immune system in combination with the use of immuno-suppressiva. Ion release has been suspected to increase the risk of DNA damage (57;88) and it was recently found that Co-Cr nanoparticles cause DNA damage across a cellular barrier (6). In addition, a reduction of circulating cytotoxic CD8+ T-cells, responsible for destroying tumor cells, was found in patients with a MOM implant (64;75). However, epidemiological studies do not

allow conclusions regarding the incidence of cancer in MOM patients (32;74;109) and will not become available anytime soon, as such studies would require thousands of patients to be followed for several decades (65).

Spleen

The spleen is an important meeting point between antigenic information transported by the blood and the immune system. Because of the central position in the bloodstream and the large blood supply of about 5% of the total blood volume per minute, the spleen will inevitably be exposed to corrosion products of MOM bearings. High concentrations of metal ions (375,000/200,000 µg/L Co-Cr) have been shown to cause alterations in spleen architecture and depletion of T4 and B cells. Therefore, the immune system and its defence against bacteria may become hampered by metal ions (34).

Liver

The liver is part of the human immune system and not only contains many immunologically active cells, but also detoxifies the body from environmental toxins. Metals can not be eliminated from tissues by metabolic degradation, but only by renal or gastrointestinal excretion (24). There is evidence from a recent animal study to suggest that Cr ions can accumulate in the liver (50). High levels of metal in the body may cause hepatocellular necrosis, as observed after acute ingestion of Cr^{4+} in humans. Clinically relevant concentrations of Cr^{4+} (10–25 µM) were found to inhibit macromolecular syntheses in the liver (53).

Immuno-competent cells

Phagocyting cells like neutrophils are vital in the host defence against infection. These 'first responders in microbial infection' are usually found in infected periprosthetic tissues. However, corrosion products of Co-Cr implant materials are reported to inhibit the rapid release of reactive oxygen species needed for bacterial killing by neutrophils (92). *In vitro* studies also showed that Co-Cr particles induce toxic effects after they are phagocytosed because of the drop in pH within the phagosome (48). Due to wear debris induced granulocyte defects, MOM patients may be predisposed to infection at the implant site (120-122).

Degradation products, either in the form of metal ions or wear particles, can complex with local proteins and induce an allergic response comparable with a delayed-type hypersensitivity response (type IV), through activation of T lymphocytes (26;38). The histological response in patients with MOM bearings is unique in its kind and is referred to as aseptic lymphocyte dominated vasculitis-associated lesion (ALVAL) (111). In addition, a statistical reduction in circulating lymphocytes, in particular of CD8 and T-cells, has been observed in patients with MOM bearings (64;75). However, beneath concentrations of 5 µg/L Co-Cr, no reduction was detected. No adverse clinical symptoms are observed in patients with increased metal ion concentrations in serum (43).

Influence of MOM degradation products on bacteria

Heavy metal toxicity

Metal ions have been used for centuries to cure infections and it is conceivable that wear products of MOM prostheses may be toxic to bacteria. There is *in vitro* and *in vivo* evidence that wear particles have toxic effects on human cells (15;51;78). *In vitro* research towards influences of Co-Cr ions on bacteria provides evidence for bacteriostatic effects (3), hypothetically involving competition with Fe for uptake in the bacterial cell. Fe is an important nutrient element required by specific microbial species which use oxidation of elemental Fe or conversion of Fe^{+2} to Fe^{+3} as an energy source for their metabolism. Inhibition of Fe-dependent metabolic activities by Co ions has been demonstrated to lead to growth retardation and cell death in *Pseudomonas aeruginosa* (56).

Within tissue cells, nanoparticles are exposed to a series of oxidative mechanisms designed to destroy the foreign body, which leads to the generation of metal ions (63). Reactions with metal ions can lead to generation of free radicals: reactive oxygen species (ROS) and reactive nitrogen species (RNS), which can, in turn, cause cellular dysfunction. ROS and RNS are known to be involved in protein oxidation, leading to their degradation, lipid peroxidation, and DNA damage. Generation of ROS and RNS may, hypothetically, cause toxicity in bacteria as well.

Bacterial growth and biofilm formation

Recent publications have evaluated the influence of Co-Cr ions and Co-Cr particles on bacterial growth. Co-Cr concentrations up to 20/9 µg/L, as reported to occur in serum, revealed no consistent influence on biofilm formation, but higher concentrations of 200,000/93,000 µg/L statistically reduced *Staphylococcus aureus* and CNS planktonic growth and biofilm formation (45), suggesting MOM bearings may be less prone to biofilm formation and subsequent infection. On the other hand, Anwar et al. (2007) showed that wear debris from MOM bearings accelerated the growth of planktonic bacteria. Aggregated particulate debris was discussed to promote growth by providing a scaffold on which biofilm can grow. In addition, it can be hypothesized that nano-sized particles scattered throughout a biofilm would enhance the strength of its structure by working as a composite scaffold on a macroscopic level. Moreover, it is also possible that detached particles embedded in a biofilm, might act as carriers of biofilm throughout the joint and body.

Heavy metal resistance

Bacteria have co-existed with abundant toxic heavy metals since the beginning of life. Therefore, it was essential for bacteria to develop mechanisms for metal-resistance. Bacterial resistance to metal toxicity is not only an environmentally important phenomenon, but also has clinical implications for metal-bacterium interactions in MOM patients. Bacterial resistance mechanisms differ widely (96) and are currently subject to extensive studies. There are enzyme oxidases and reductases to convert metal ions from more toxic into less toxic (14;22;52;62). There is also the possibility of binding heavy metals in the bacterial cell wall (54). Blocking of cellular uptake is also an option by altering the uptake pathway. Once the toxic heavy metal has reached the intracellular cytoplasm, it can be pumped out again by a high efflux system (71;73). Efflux pumps are the major currently known group of resistance systems.

Co-selection of antibiotic and metal resistance

There is growing concern that metal contamination may function as a selective agent in the proliferation of antibiotic resistance (4). It is hypothesized that

antibiotic-resistant bacteria can be maintained in the environment owing to the co-regulation of resistance pathways (4;115). These co-selection mechanisms include co-resistance (different resistance determinants present on the same genetic element) and cross-resistance (the same genetic determinant responsible for a conjoint resistance to antibiotics and metals). Co-resistance in clinical isolates of *Staphylococcus* species has been described against multiple metals and antibiotics (103), but the most common co-resistance involved Cr, Pb and penicillin. Co-Cr increased the sensitivity of staphylococci to penicillin, while sensitivity to tetracycline decreased (69). The mechanisms behind this co-selection are currently investigated. However, it has been found that the reduction in permeability of bacteria causes Co, and Ag resistance, via a similar mechanism which is responsible for inhibiting ß-lactam, ciprofloxacin, tetracycline and chloramphenicol from entering the bacterium (86;97). On top of this, reduced permeability, a rapid efflux mechanism, is also used to prevent Co, Cu, Zn, Cd and Ni from entering the micro-organism, similar to the resistance mechanism of ß-lactam, tetracycline and chloramphenicol (61;72). The clinical incidence of co-selection mechanisms of resistance factors in pathogenic bacteria for antibiotics and heavy metals and their clinical implications remain unknown.

Conclusions

Unfortunately, long-term clinical data on infection rates for MOM bearings are not yet available and therefore actual clinical influences on infection could not be evaluated. To assess the clinical influence of bearing type on infection risk, studies will require thousands of patients to be followed for several decades. Such data may soon become available from national joint registries and their evaluation will shed light on the net influence of bearing type on infection risk. However, this review suggests that wear particles and its corrosion products may influence the risk of infection by hampering the immune system, inhibiting or accelerating bacterial growth and by a possible antibiotic resistance and heavy metal co-selection mechanism. Whether this influence results in an increase or decrease of clinical infection rates has not yet been investigated.

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Chapter 3

Metal-on-metal bearings in hip arthroplasties: Influence of Co-Cr ions on bacterial growth and biofilm formation

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Abstract

Background

Metal-on-metal (MOM) bearings involving cobalt-chromium (Co-Cr) alloys in total hip arthroplasties are becoming increasingly popular due to their low wear. Consequences of corrosion products of Co-Cr alloys are for the most part unclear, and the influence of cobalt and chromium ions on biofilm formation has never been studied. Therefore the aim of this study was to evaluate how Co-Cr ions affect bacterial growth, biofilm formation and architecture.

Methods

A collection of clinically isolated and commercially available bacterial strains were exposed to Co-Cr concentrations as found in serum and higher as found in adjacent tissue.

Results

Planktonic growth of bacteria was inhibited by concentrations of 200,000/93,000 µg/L Co-Cr. Co-Cr concentrations up to 20/9.3 µg/L as reported to occur in serum revealed no consistent influence on biofilm formation, but higher concentrations of 200,000/93,000 µg/L significantly reduced *Staphylococcus aureus* and CNS biofilm formation. As indicated by confocal laser scanning microscopy, no dead bacteria were encountered in the biofilms, and the metal ion concentrations used must be classified as growth-inhibiting and not bactericidal.

Conclusion

Long-term clinical data on infection rates for Co-Cr MOM-bearings are not yet available, but the current results suggest that Co-Cr ions may yield these prostheses less prone to biofilm formation and subsequent infection.

Introduction

Total hip replacement is a highly successful procedure with a regain of a relatively high quality of life and an almost instant pain relief. Success of the procedure resulted in younger cohorts of patients. Many of these younger patients want to return to a high level of activity and seek an implant that provides durability. Larger femoral heads were indicated (10)], but this tended to cause excessive wear in conventional prostheses (metal ball connected to large stem and a cup with polyethylene interface). Polyethylene wear and debris is a suspected cause of osteolysis around the implant (27;44), which led to the development of alternative bearings lacking a polyethylene/metal interface, like the recently reintroduced metal-on-metal (MOM) bearings. In contrast to metal-on-polyethylene bearings, wear rates of MOM-bearings turned out to be impacted in a positive way by increasing the head size (1;22), yielding 20-100 times less debris than in traditional metal-on-polyethylene bearings (34). The remarkably low wear of MOM-bearings has led to a rapidly increasing popularity of MOM-articulation in the treatment of young and active patients (40).

Although mid- and long-term clinical results of MOM-bearings appeared to have demonstrated excellent durability, recent studies show that there is at least one MOM-bearing system with periprosthetic osteolysis and aseptic loosening, which is possibly associated with hypersensitivity to metal debris (19;24). Additionally, MOM-articulations are not completely biologically inert, since they produce metal particles that can be found in e.g. blood and urine. These particles tend to corrode and serum levels of metal ions, mainly cobalt and chromium, become elevated (5;11;30;31;35;43). Cobalt and chromium are usually eliminated only slowly from the body by urine, and chromium is even retained in the body's tissues (9;31). These high cobalt and chromium serum concentrations may have toxic effects which include the increase of bone resorption, and theoretical risks of delayed-type hypersensitivity, organ toxicity and altering of cell homeostasis (15;16;21;23;36;37). Furthermore, cobalt and chromium have been shown to be carcinogenic and mutagenic in human and animal models (3;28;39;42), which implies that systemic toxicity and cancer risk may be possible disadvantages of MOM-articulation.

Alongside these possible disadvantages, it is also conceivable that the risk of infection is influenced by metal ions. Infection still remains a significant complication following total hip replacement and as a conservative estimate, affects about 1-2% of all patients during the lifetime of an implant (7). In case of infection, bacteria adapt a biofilm mode of growth on the surface of the prosthesis, which represents a basic survival mechanism of the organisms (14) to external (500 to 5000 times increased antibiotic resistance (13;26)) and internal environmental factors (the host immune system). The associated increased antibiotic resistance of biofilms causes major difficulties in patient treatment. Removal and replacement of an infected implant is usually required to eliminate the infection with accompanying trauma and increased costs to the health service (6;8;38). Copper and zinc are known for their bactericidal properties and impact on biofilm formation (17), but no research efforts have been undertaken towards the specific influence of the cobalt-chromium ion combination on biofilm formation, despite extensive other studies into MOM-bearings (12;32;33).

The aim of this *in vitro* study is to evaluate the influence of cobalt and chromium ions on bacterial growth, biofilm formation and architecture for a collection of clinically isolated and commercially available bacterial strains.

Materials and methods

Bacterial strains

Gram-positive organisms account for most bacteria found in infected hip arthroplasties. Coagulase negative staphylococcus (67%) was found to be the predominant organism, although *Staphylococcus aureus* (13%) is gaining importance (29). Therefore, a total of 13 staphylococcal strains were used in this study (Table 1), chosen to represent their frequency of occurrence in clinical infection. Eight strains were isolated with extensive biomaterial culturing (25) from explanted metal-on-polyethylene joint prostheses from individual patients with septic loosening and retrieved during revision surgery (Department of Orthopaedic Surgery at the University Medical Center Groningen, The Netherlands) and five additional strains were of ATCC origin.

Cobalt and chromium ions

Metal ion concentrations of 2/0.93; 20/9.3; 20,000/9300; 200,000/93,000 µg/L Co-Cr were applied throughout this study. The lowest Co concentration of 2 µg/L was inline with previously found Co serum concentrations (11;12) and the proportion Co-Cr in this study was chosen similar to most MOM-bearings currently used in Europe (± 61% Co and 29% Cr). The second-lowest level of 20/9.3 µg/L Co-Cr was chosen to represent higher serum levels, described in the literature. Higher concentrations of metal ions were used, since the local concentration of metal ions in the synovial fluids is expected to be much higher than the serum concentrations.

Based on a previously described method (4), 0.847 mg cobalt salt ($CoCl_2.6H_2O$, Sigma) and 0.475 mg chromium salt ($CrCl_3.6H_2O$, Merck) were dissolved in 10 mL tryptone soya broth (TSB) (Oxoid, Basingstoke, United Kingdom). These samples contained 200,000/93,000 µg/L Co-Cr and were diluted with broth to reach the concentrations and Co-Cr proportions needed for the experiments.

Planktonic growth evaluation

Three *S. aureus* strains and two CNS strains were randomly chosen for growth curve evaluation. These isolates were routinely cultured from frozen stock on blood agar plates at 37°C for 24 h. Precultures were inoculated with a single plate colony, grown in 10 mL TSB and incubated aerobically overnight at 37°C. From the resulting suspension, 1 mL was inoculated overnight with 45 mL TSB, 45 mL TSB with 20,000/9300 µg/L Co-Cr or 45 mL TSB with 200,000/93,000 µg/L Co-Cr. The absorbance at 600 nm (A_{600}) was determined using a spectrophotometer. All growth curve experiments were performed twice with separately cultured bacteria.

Biofilm formation in microtiter plates

All strains mentioned in Table 1 were routinely cultured from frozen stock on blood agar plates at 37°C for 24 h. Precultures were inoculated with a single colony from plate grown in 10 mL TSB and incubated overnight aerobically at 37°C. A bacterial suspension of 200 µL (2 µL preculture and 198 µL fresh TSB) supplemented with different concentrations of metal ions, was used to inoculate a well of a 96-well polystyrene flat-bottomed tissue culture plate (Falcon, Becton Dickinson, Oxnard, CA) for biofilm formation. After 24 h at 37°C, the growth media and planktonic cells

in the 96-wells plates were removed from the biofilms by carefully replacing the volume of the wells twice with 200 µL 10 mM potassium phosphate, pH 7.0 by pipetting, while taking care that air-liquid interface passages over the biofilms were avoided. The wells were subsequently stained with 200 µL 1% crystal violet. After 30 min, excess stain was replaced with 200 µL demineralised water as described above, and the crystal violet was dissolved in 200 µL of ethanol-aceton (80:20 vol/vol). The absorbance at 575 nm (A_{575}) was determined using a microtiter plate reader (Fluostar Optima) to determine the amount of crystal violet, as a measure of biofilm growth. The influence of Co-Cr ions on biofilm formation was evaluated by measuring the percentage of growth stimulation/reduction according to

$$\text{growth stimulation / reduction} = \frac{(A_{575\ \text{presence Co-Cr}} - A_{575\ \text{absence Co-Cr}})}{A_{575\ \text{absence Co-Cr}}} \text{ X } 100\%$$

Thus inhibitory effects of the presence of Co-Cr ions appear as negative numbers in the outcome parameter. All experiments included six replicate wells and were performed three times with separately cultured bacteria.

Biofilm architecture determination by confocal laser scanning microscopy (CLSM)

Two of the five strains used for growth curve evaluation, *S. aureus* 7388 and a CNS 5147, were used for visualizing biofilm architecture. These isolates were routinely cultured from frozen stock on blood agar plates at 37°C for 24 h. Precultures were inoculated with a single colony from plate grown in 10 mL TSB and incubated overnight aerobically at 37°C. From the above-mentioned bacterial suspension, 25 µL was inoculated with respectively 3 mL TSB, 3 mL TSB with 2/0.93 µg/L Co-Cr or 3 mL TSB with 200,000/93,000 µg/L Co-Cr in a 6-wells polystyrene tissue culture plate (Costar). After 24 h of incubation at 37°C, biofilms were stained with calcofluor white (Bayer) to visualize extracellular polymeric substance (fluorescent blue), and with LIVE/DEAD Baclight viability kit (Molecular Probes Inc., Eugene, Oreg.), to visualize live (fluorescent green) and dead (fluorescent red) bacteria. After 15 min incubation in the dark, confocal images were collected using a Leica TCS-SP2 microscope with a 40x water objective. Images were obtained at 1 to 2 µm intervals down through the biofilm and the number of images, therefore, corresponded with

the thickness of the biofilm. The CLSM experiments were performed twice with separately cultured bacteria.

Statistical analysis

Differences in optical densities of biofilms grown in the absence and presence of metal ions were analyzed for significance by the Student t-test for paired samples. A 95% ($p < 0.05$) confidence interval was applied for statistical significance.

Results

Planktonic growth

Figure 1 summarizes the planktonic growth of *S. aureus* 7388 and a CNS 5147 in the absence and presence of different concentrations of Co-Cr ions. Clearly for these two strains as well as for the other three strains involved in planktonic growth experiments (data not shown), planktonic growth was not significantly influenced by Co-Cr as compared with the control when the ion concentrations were less than 20,000/9300 µg/L Co-Cr, but at the highest concentration of 200,000/93,000 µg/L Co-Cr all *S. aureus* and CNS strains showed significant growth reduction.

Biofilm formation

Table 1 summarizes the effects of different concentrations of Co-Cr ions on biofilm formation of the *S. aureus* and CNS strains involved. Whereas most isolates show growth reductions that increase with increasing Co-Cr concentrations, some strains are clearly stimulated in their growth at low metal ions concentrations (*S. aureus* ATCC 12600 and *S. epidermidis* ATCC 35984). At the highest metal ion concentrations, however, all strains are reduced in their growth. When averaged over all isolates of a given species, it becomes clear that *S. aureus* and CNS are inhibited in their growth when Co-Cr concentrations are above 200,000/93,000 µg/L. At that concentration, CNS is slightly more affected than *S. aureus*.

Biofilm architecture

CLSM images of the *S. aureus* 7388 and CNS 5147 biofilms grown in the absence and presence of Co-Cr ions revealed a decrease in the number of live bacteria due

to the presence of Co-Cr ions (Figure 2). Sectional analysis of each biofilm layer (about 1 µm in thickness) made it possible to demonstrate the three-dimensional structure of biofilms, and revealed that the biofilms formed in the absence of Co-Cr ions had a thickness of respectively 42 µm (*S. aureus*) and 35 µm (CNS). While in the presence of the highest concentration Co-Cr ions (200,000/93,000 µg/L) biofilm thickness and density is remarkably reduced to 15 µm and 8 µm, for *S. aureus* and CNS, respectively, confirming that CNS is slightly more affected. Neither dead bacteria nor slime were observed, regardless of the absence or presence of Co-Cr ions or the strain involved.

Table 1. The percentage of growth stimulation/reduction after 24 h metal ion exposure.

	Co/Cr ion concentration (µg/L)			
	2/0.93	20/9.3	20,000/9300	200,000/93,000
Staphylococcus aureus				
Staphylococcus aureus 5296	4%	-4%	-8%	-12%
Staphylococcus aureus 7388	-3%	-7%	-10%*	-17%*
Staphylococcus aureus ATCC 12600	15%	9%	-15%	-35%*
Staphylococcus aureus ATCC 25923	-4%	0%	0%	-11%*
Staphylococcus aureus ATCC 51153	4%	4%	0%	0%
Mean (SD = 8%)	**3%**	**0.4%**	**-7%**	**-15%***
CNS				
CNS 7391	4%	-6%	-20%	-45%*
CNS 5115	-9%	-7%	-11%	-20%
CNS 5295	7%	11%	0%	-4%
CNS 7319	-4%	0%	-8%	-8%
CNS 7349	-4%	4%	-32%*	-47%*
CNS 5147	-9%	-2%	-9%	-38%*
S. epidermidis ATCC 35984	10%	21%*	5%	-28%*
S. epidermidis ATCC 14990	-3%	0%	3%	-21%*
Mean (SD = 13%)	**-1%**	**3%**	**-9%**	**-26% ***

* indicates a significant difference versus growth in the absence of metal exposure, i.e. 0% growth stimulation/reduction ($p < 0.05$). Values are averages including standard deviations from three experiments with separately cultured bacteria, yielding an average mean standard deviation of 11%. Note that mean values in bold represent the mean including standard deviations over the collection of isolates involved. Growth reductions appear as negative numbers.

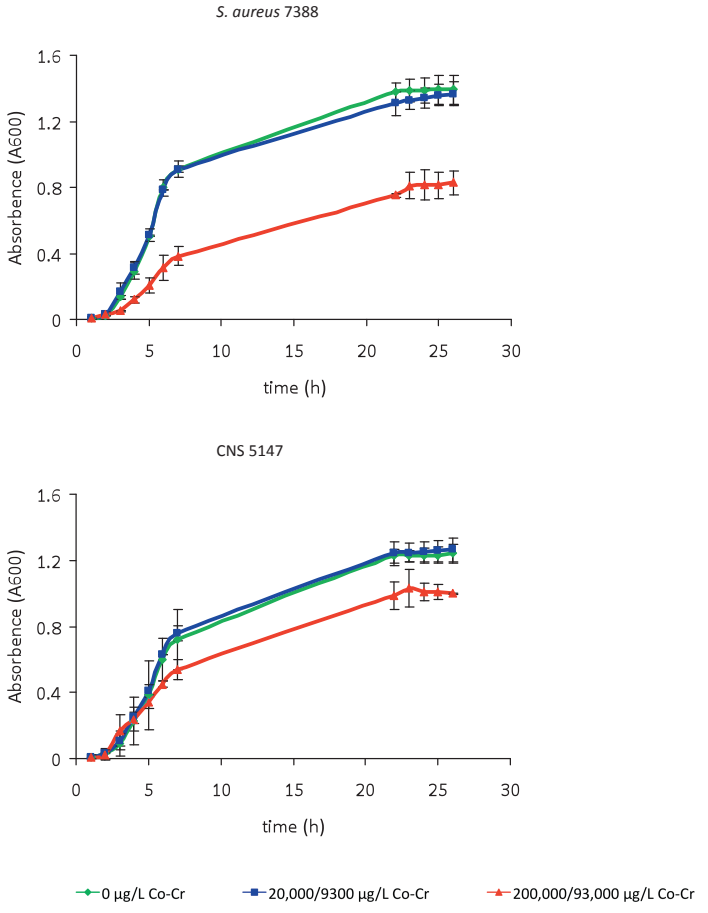

Figure 1. Growth curves of two clinically isolated strains: *S. aureus* 7388 and CNS 5147 in the absence and presence of metal ions. Error bars indicate the standard deviation of the mean calculated from two growth curve experiments with separately cultured bacteria.

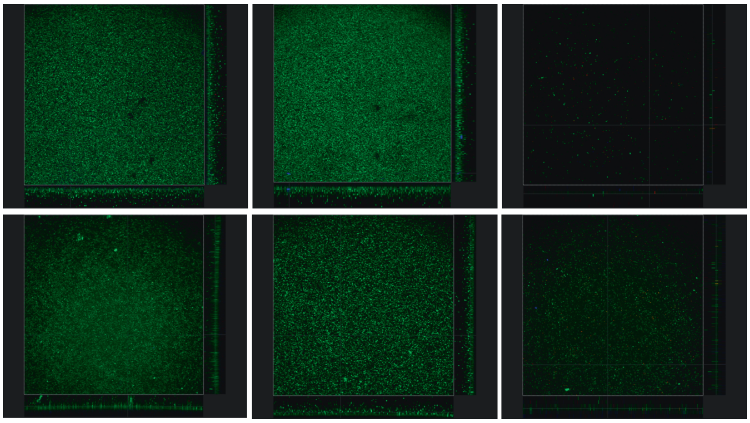

Figure 2. CLSM images (375 x 375 µm) of *S. aureus* 7388 (top) and CNS 5147 (bottom) biofilms, grown in the absence (left) and presence of 2/0.93 µg/L (middle) and 200,000/93,000 µg/L (right) Co-Cr ions.

Discussion

MOM-bearings for joint arthroplasty have regained popularity in the treatment of young patients because they offer wear rates low enough to prevent the bearing from wearing out in a lifetime, but little is known about the way infectious microorganisms behave toward the Co-Cr surfaces involved in MOM-bearings. Recently, Anwar et al. showed that wear debris from MOM-bearings accelerate the growth rate of planktonic bacteria [41]. To our knowledge, this is the first study that focuses on the influence of cobalt and chromium ions on biofilm formation.

An intriguing novel finding from this study was that biofilm formation and planktonic growth were inhibited by a high dose of chromium and cobalt ions. The highest concentration of metal ions (200,000/93,000 µg/L Co-Cr) used, reduced biofilm formation by 15% and 26% with respect to a control for two collections of *S. aureus* and CNS strains, respectively. The lower metal concentrations revealed no consistent influence on biofilm nor on planktonic growth. CLSM images of the biofilm confirmed the results retrieved by light absorbance and showed that biofilm thickness and density were only affected by exposure to the highest concentration Co-Cr. The highest metal ion concentration caused a reduction in biofilm thickness of more than 50%.

The exact role of either cobalt or chromium in staphylococcal biofilm formation is still unclear and hypothetically involves competition with Fe for uptake in the cell. Iron is an important nutrient element required by the bacterial metabolism, and interference with its uptake could provide an effective mechanism to contain infection. This suggestion is confirmed by a study on the effect of cobalt on *Pseudomonas aeruginosa* (20), demonstrating inhibition of iron-dependent metabolic activities of the bacterium leading to growth retardation and cell death.

Cobalt chromium alloys had not been available before the nineteen fifties and it was at that moment when the first designs of MOM-bearings were described. Initially, infection rates of early MOM-bearings developed in the nineteen sixties, such as the McKee-Farrar arthroplasty were high and ranged from 0% to 6% with antibiotic prophylaxis and from 0.5% to 11% without, but at that time in many centres no clean air enclosures were used (2). However, the durability of these designs was quite poor and at present the durability of the bearing surfaces has been improved by appropriate surface finishes and forging processes. Preliminary data over short follow-up times of these newly developed MOM-bearings show lower infection rates than of the early designs. Milosev *et al*. reported six revisions because of infection in a cohort of 640 total hip replacements after a 7.1 year follow up (24). In addition, Korovessis *et al*. reported 3 infections in a consecutive series of 217 total hip replacements after 6.4 years (19). However, no large, long-term outcome studies are presently available.

Although the reductions in biofilm formation observed in the present study seem to be in line with the few clinical data on infection rates of MOM-bearings, it must be acknowledged that reliable information about the exact local concentrations of Co-Cr around prostheses is not available. However, in local antibiotic treatment it is recognized that local antibiotic concentrations can become up to 5000 times higher than serum levels (41), which suggest that Co-Cr concentrations around a MOM-bearing may be as high as 100,000/46500 µg/mL. In addition, serum levels of metal ions have demonstrated great variability from patient to patient (18). Moreover, local concentrations of Co and Cr ions in the synovial fluids will probably exceed these serum levels significantly, particularly in

poorly engineered implants or in case of increased wear rate because of malpositioning of the components, impingement, or loosening.

In conclusion, planktonic bacterial growth, biofilm growth and thickness were significantly reduced by Co-Cr concentrations of 200,000/93,000 µg/L, which are higher than observed in serum, but not unlikely around a prosthesis or in synovial fluid. This suggests that MOM-bearings may be less prone to biofilm formation and subsequent infection.

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Chapter 4

Influence of Co-Cr particles and Co-Cr ions on the growth of staphylococcal biofilms

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Submitted

Abstract

Background

In the last decades, hip prostheses with a metal-on-metal (MOM) bearing have been implanted by orthopedic surgeons worldwide. However, concerns are now raised towards the metal particles and degradation products released by MOM-bearings into surrounding tissue, although effects of Co-Cr wear on infection are also unknown. Therefore, we here determine the viable volumes of staphylococcal biofilms formed on polystyrene in the absence and presence of Co-Cr particles and Co-Cr ions.

Methods

Three clinically derived and two commercially available staphylococcal strains were grown in the presence of 2 mg/mL Co-Cr particles or 1000/500 µg/L Co-Cr ions derived from Co-Cr salts or from particle supernatant, under static and dynamic growth conditions. A dynamic model simulates the conditions that apply for biofilm formation in the human body, as synovial fluid in mobile patients with hip prostheses is in constant motion with accompanying shear rates. Images of 24 h old biofilms were made with confocal laser scanning microscopy and analyzed with the mathematical computer program COMSTAT, yielding the biovolume of a biofilm. X-ray photoelectron spectroscopy was performed on the particles to study their elemental surface composition.

Results

Most isolates showed a tendency of reduced biofilm growth in the presence of Co-Cr particles compared to growth during exposure to metal ions, but this was only significant in one strain under the dynamic growth condition (*Staphylococcus aureus* 7388). Characterization of the outer surface of the particles revealed a Co-Cr oxide layer enriched by Molybdenum relative to the bulk concentration.

Conclusion

MOM bearings produce metal particles which were found to possess antibacterial characteristics under dynamic growth conditions. Further research is needed towards the clinical relevance of this finding.

Introduction

Large metal-on-metal (MOM) articulations have been implanted frequently in active and young patients, mainly due to their reduced dislocation rates (18;26) and low wear characteristics (1;27). However, active MOM patients accumulate nearly 1 mg of nano-sized wear particles per year (2;25). Concerns regarding the production of wear particles and its corrosion products have led to questioning of the indications for Co-Cr alloy bearing use (5). Recently, the Healthcare products Regulatory Agency in the United Kingdom has sent out an alert to hospitals and doctors regarding adverse soft tissue reactions to wear of MOM implants. In addition, it is conceivable that the vast amount of particles influence infection severity.

High concentrations of Co-Cr ions have been found to yield bacteriostatic effects on planktonic grown bacteria and biofilm formation (15). On the other hand, particulate Co-Cr debris was shown to promote planktonic bacterial growth (4). However, these studies were performed under static conditions. A static set-up does not account for shear during biofilm formation, as occurring between implant surfaces and synovial fluid of mobile patients. Therefore, the aim of this study was to compare the effect of Co-Cr particles and Co-Cr ions on viable biofilm volume in a static and dynamic model, simulating conditions around an implant in the human body.

Materials and methods

Co-Cr particles and characterization

Particles were derived from cast Co-Cr alloy Micro-Melt® dust (Carpenter Powder Products, Wyomissing, PA), obtained by courtesy of Biomet (Warsaw, IN). The Co-Cr alloy according to ISO 5832-4 standards, contains Co-Cr, but also molybdenum (Mo), nickel, iron, silicon and manganese alloying elements (see Table I).

X-ray photoelectron spectroscopy (XPS) was performed with a S-probe spectrometer (Surface Science Instruments, Mountain View, CA) equipped with an aluminium anode (10 kV, 22 mA) and a quartz monochromator. The binding energy scale was calibrated to the C_{1s} peak at 284.8 eV. The experimental peaks were integrated after linear background subtraction. Elemental surface compositions

were calculated from the integrated peak areas employing instrumental sensitivity factors as supplied by the manufacturer and expressed in atom%. Elemental depth profiling of the particles was done by Ar^+-ion sputtering using a VG ion gun AG 2.1.

Particle size distributions were measured with a Sympatec HELOS compact KA laser diffraction apparatus (Sympatec GmbH, Clausthal-Zellerfeld, Germany), using a RODOS dry powder disperser (at 3.0 bar). A lens of 200 mm was used and calculations were based on the Fraunhofer diffraction theory.

Wear rates of removed MOM implants for early aseptic loosening have been reported as low as 4.6 mg/year (23) with an average amount of synovial fluid in a hip of 2.5 mL (range, 0.1-5.6) (16;20). Based on these data, a particle concentration of 2 mg/mL was used throughout our experiments, assumed to reflect a concentration within the range of clinically expected values.

Metal ion concentration derived from salts and supernatant

To establish diluted metal ion concentrations of Co-Cr particles, three samples of autoclaved tryptone soya broth (TSB) (Oxoid, Basingstoke, United Kingdom) with 2 mg/mL particles were filtrated to measure Co-Cr concentrations in the supernatant with atomic absorption spectroscopy, by courtesy of Thermo Fischer (Waltham, MA). Supernatant samples revealed concentrations of 1100 µg/L cobalt (range 943-1440 µg/L) and 409 µg/L chromium (range 357-501 µg/L). Metal salt solutions were subsequently prepared accordingly with a concentration of 1000 µg/L cobalt and 500 µg/L chromium. Based on a previously described method (3), 4.037 mg cobalt salt ($CoCl_2.6H_2O$, Sigma-Aldrich, Zwijndrecht, Netherlands) and 2.564 mg chromium salt ($CrCl_3.6H_2O$, Merck, Darmstadt, Germany) were dissolved in 10 mL TSB. These samples contained 100,000/50,000 µg/L Co–Cr and were diluted with broth to reach the concentrations described above.

Bacterial strains

A total of three *Staphylococcus aureus* strains, one *Staphylococcus epidermidis* and one coagulase-negative staphylococcal (CNS) strain were involved in this study, representing predominant organisms in infected bone and joint samples (8;22). Three strains were isolated using extensive culturing from explanted metal-on-

polyethylene joint prostheses from individual patients with septic loosening (21). The remaining two additional strains were of ATCC origin.

Biofilm formation

Isolates were routinely cultured from frozen stock on blood agar plates aerobically incubated at 37°C for 24 h. Precultures were inoculated with a single colony from an agar plate in 10 mL TSB and incubated overnight aerobically at 37°C. From this preculture, 30 µL was inoculated in respectively 3 mL TSB (control), 3 mL TSB with 2 mg/mL Co-Cr particles, 3 mL TSB with 1000/500 µg/L Co-Cr ions derived from metal salts and 3 mL TSB with supernatant of 2 mg/mL Co-Cr particles in a 6-wells tissue culture polystyrene plate (Greiner, Frickenhausen, Germany). Each set of experiments was performed in triplicate with separately cultured bacteria, and grown under static and dynamic (150 rpm) growth conditions.

Biofilm imaging with confocal laser scanning microscopy (CLSM)

After 24 h of incubation at 37°C, biofilms were washed gently, with phosphate buffer saline (PBS; pH 7.0), ensuring the biofilm remained intact. To visualize live bacteria (fluorescent green), all wells were stained with LIVE/DEAD *Bac*Light viability kit (Molecular Probes Inc., Eugene, OR). Due to reflecting particles at an excitation wavelength of 488 nm and emission filter settings of 600 nm and higher, it was not possible to determine the amount of dead bacteria (fluorescent red) on particles. After 15 min of incubation in the dark, representative CLSM images were collected using a Leica TCS-SP2 microscope with a water objective.

Quantification of biofim formation with COMSTAT

CLSM images were analyzed with the mathematical computer program COMSTAT, yielding the biovolume of a biofilm, as described earlier (14). Biovolume ($\mu m^3/\mu m^2$), defined by biomass pixels in all images of a stack multiplied by the voxel size and divided by substratum area, represents the overall volume of a biofilm. In this study, only the viable biovolume was determined.

Scanning electron microscope (SEM) samples

To visualize the biofilm on the Co-Cr particles with SEM, biofilm of *S. aureus* ATCC 12600 was grown under static and dynamic growth conditions, as described above. Samples were fixed in 2% glutaraldehyde/0.1 M cacodylatebuffer for at least 24 h at 4°C. A post-fixation was performed using 1% osmiumtetroxide in 0.1 M cacodylatebuffer for 1 h at room temperature. After several washings with water, samples were dehydrated through an ethanol series. After the final 100% ethanol step, samples were treated with ethanol/tetramethylsilane followed by pure tetramethylsilane (both 15 min at 0°C) and subsequently air dried. After sputter coating the samples with approximately 5 nm Pd/Au, imaging was performed at 2 kV using a JEOL JSM6301 scanning microscope.

Statistics

Differences in biovolumes of biofilms grown in the absence and presence of metal particles or metal ions were analyzed for significance by ANOVA and followed by a Mann-Whitney U-test. A 95% ($p < 0.05$, two-tailed) confidence interval was applied for statistical significance.

Results

Co-Cr particles

The Co-Cr and oxygen depth profile (Table I) of the particles was typical for a surface oxide layer; the oxygen signal decreased towards the bulk metal. The Co signal was similar to the Cr one at the outermost surface and increased strongly after Ar^+-ion sputtering, whereas the Cr signal only increased slightly. Particle surfaces appeared enriched by Mo relative to the bulk concentration prior to sputtering. After sputtering, Mo was present in lower concentrations. Carbon contamination, as present in all XPS measurements, remained to be present after sputtering. Co-Cr particle size distributions are presented in Table II. Particles were found to have an average size of 40 µm.

Table I. Chemical composition and surface characterization of Micro-Melt® CCM+.

Elements	Micro-Melt® CCM+ bulk composition		XPS surface composition (Atom%)	
	Weight %	Atom%	Before sputtering	After sputtering
Cobalt, Co	balance to 100	60.0	7.3	49.0
Chromium, Cr	29.5	32.4	7.6	9.4
Molybdenum, Mo	6.6	3.9	4.5	2.8
Nickel, Ni	0.1	0.1		
Iron, Fe	0.3	0.3		
Silicon, Si	0.7	1.4		
Manganese, Mn	0.7	0.7		
Carbon, C	0.2	1.1	38.7	24.1
Oxygen, O	-		42.0	14.7

Table II. Volume size distribution of Co-Cr particles derived from Micro-Melt ® dust.

Cumulative distribution (%)	Particle size beneath (µm)
10	18
25	32
50	40
75	49
90	58
99	71

Table III. Viable biovolume ($\mu m^3/\mu m^2$) of a collection of three clinically derived staphylococcal strains and two ATCC strains, grown under static and dynamic conditions and in the absence and presence of metal ions and particles.

		Control	Metal ions (salt)	Metal ions (supernatant)	Metal particles
Static	*S. aureus* ATCC 12600	11	12.8	18.8	7.6
	S. aureus 7388	13.8	6.8	8.9	4.1
	S. aureus 5296	13.5	8.9	12.5	18.3
	S. epidermidis ATCC 35984	20.9	14	15.7	15.7
	CNS 7391	11.3	10.7	4.9	8.2
Dynamic	*S. aureus* ATCC 12600	4.1	7.1	2.4	1.7
	S. aureus 7388	4.1	3.8	0.9*,**	0.2*,**
	S. aureus 5296	7.6	1.5	13.3	3.7
	S. epidermidis ATCC 35984	6.3	4.2	2.2	2.7
	CNS 7391	2.2	4.2	2.8	0.7**

*Indicates a significant difference in viable biovolume compared to the control, ** compared to metal ions (salt) ($p < 0.05$). Values are averages from three experiments with separately cultured bacteria, yielding an average mean standard deviation of 22%.

Biofilm formation

Table III summarizes the effects of Co-Cr ions derived from particles and from metal salts and Co-Cr particles on staphylococcal biofilm formation. Most isolates show a tendency of biofilm growth reduction when grown in the presence of Co-Cr particles compared to the control (no metal ions), but this is only significant for *S. aureus* 7388 under dynamic growth conditions. CNS 7391 and *S. aureus* 7388 growth was inhibited significantly more by metal particles than by metal ions derived from Co-Cr salts. Moreover, metal ions derived from particles were inhibiting growth of *S. aureus* 7388 significantly more compared to metal ions derived form Co-Cr salts.

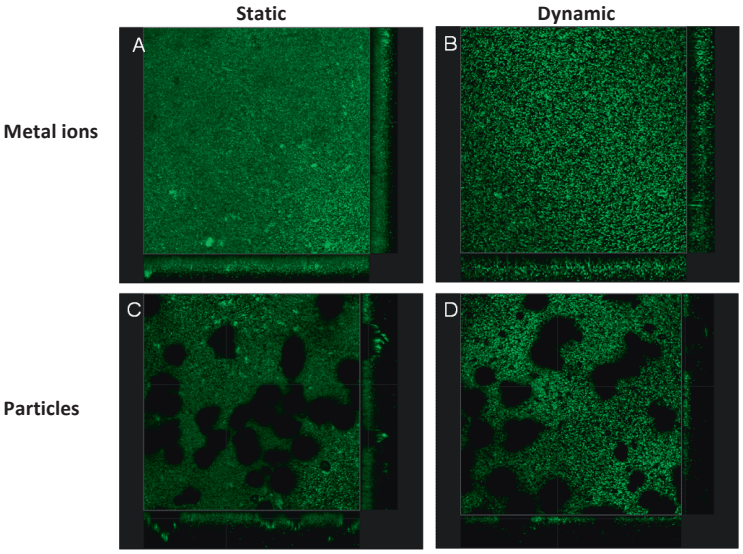

Figure 1. CLSM images (375 x 375 µm) of *S. aureus* 5296 biofilm, grown under static (A & C) and dynamic conditions (B & D).

Sectional (about 1 µm in thickness) analysis of each biofilm made it possible to reconstruct the biofilms. Side views of CLSM images of *S. aureus* 5296 biofilms illustrate that the biofilm is far thinner in the presence of Co-Cr particles compared to the control (Figure 1). Biofilm architecture in the absence of particles,

whether it was in the presence or absence of metal ions, was comparable for all strains tested.

Figure 2 shows electron micrographs of Co-Cr particles which were incubated with bacteria under dynamic (Figure 2A) and static growth conditions (Figure 2B). When grown under dynamic conditions, only a few bacteria adhered to the particles, whereas under static growth conditions, the surface of the particles is covered with bacteria.

Static

Dynamic

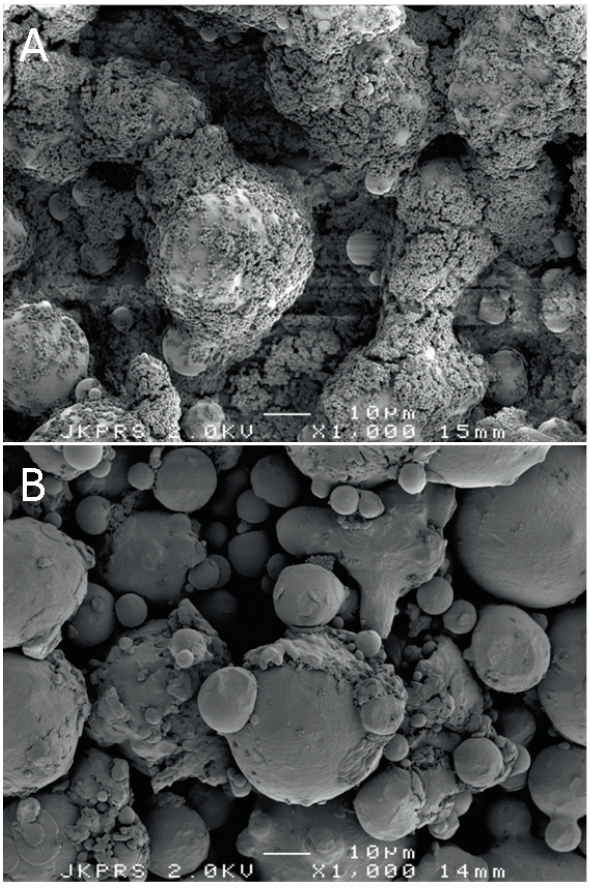

Figure 2. SEM images of Co-Cr particles covered with a biofilm of *S. aureus* ATCC 12600 after growth under static (A) and dynamic (B) conditions.

Discussion

Co-Cr particles and Co-Cr ions alone appeared to have no significant influence on biofilm formation under static growth conditions. Changing the experimental conditions from static to dynamic decreased the ability of bacteria in general to produce biofilm, with systematic differences found between individual strains. In addition, the dynamic growth condition indicated bacteriostatic effects of Co-Cr particles for *S. aureus* 7388. Thus, shear stress appears to have great influence on biofilm formation, as also seen by other authors (9).

The dynamic growth conditions provide a model for biofilm formation, simulating *in vivo* conditions in mobile patients with a constant synovial fluid flow. However, the standard deviation over the data in Table III is rather large (22% on average), which impedes drawing of statistically significant conclusions for each strain. The average biovolume over all strains of control biofilms formed in the absence of metal ions or particles was 14.1 ± 7.2 $\mu m^3/\mu m^2$ (static growth condition) and 4.8 ± 3.0 $\mu m^3/\mu m^2$ (dynamic growth condition), which constitutes a statistically significant difference ($p < 0.01$) between static and dynamic growth conditions. In the presence of the Co-Cr particles and compared to the control, biovolume was reduced to 10.8 ± 8.7 $\mu m^3/\mu m^2$ under static ($p > 0.05$) and to 1.8 ± 2.5 $\mu m^3/\mu m^2$, under dynamic ($p < 0.01$) growth conditions. Biovolumes as averaged over all strains were not significantly ($p > 0.05$) affected under dynamic growth in the presence Co-Cr ions derived from salt (4.3 ± 6.0 $\mu m^3/\mu m^2$) or supernatant from Co-Cr particles (4.2 ± 4.7 $\mu m^3/\mu m^2$)). Although we are concerned about the incidence of large standard deviations throughout our study, this method has been able to differentiate biofilm growth under static and dynamic conditions as averaged over different strains.

It is interesting that under dynamic growth, biofilm formation is inhibited in the presence of Co-Cr particles compared to the control, while this inhibitory effect is not seen under static growth conditions. This difference between the dynamic and static conditions indicates that bacteria are much more vulnerable under dynamic conditions due to their inability to form a thick biofilm (6). Metal ion concentrations in direct vicinity of Co-Cr particles may be high enough to reduce biofilm formation by more or less single adhering bacteria (dynamic growth condition), but below the inhibiting concentration for bacteria in a thick, full-grown

biofilm (static growth condition). In addition, the Co-Cr ion mass transfer into the biofilm may be larger under the dynamic condition with a larger Co-Cr ion concentration gradient through the biofilm than under a static one.

The particles used in this study are not within the nanometer range as found *in vivo*, possessing ranges between 40-120 nm (7;19). Smaller particles may release significantly more ions due to their larger area to volume ratio and thus yield more pronounced effects than seen here. However, many clinically retrieved Co-Cr wear particles are in the micrometer size range (28;29), as in the current study. Frequently micrometer sized particles are described as irregularly shaped, fragmented or clustered, suggesting these particles to be aggregates of smaller particles. Unfortunately, nanometer sized particles are scarce and not yet available in large quantities needed for *in vitro* research.

Trace ions of alloying elements were not responsible for biofilm inhibition as no significant effect was found towards averaged biofilm volumes when grown during exposure to particle derived, supernatant metal ions. The exact role of Co-Cr ions in biofilm inhibition is still unclear. Staphylococcal strains possess Co-Ni permeases which facilitate influx of Co and Ni (13). Ni is known for its bactericidal effect in staphylococcal strains (4). However only 0.30 % of the particle alloy used consists of Ni (Table I). As for Co, this metal is suspected to display toxic effects due to its involvement in competition with iron for uptake in the cell in *Pseudomonas aeruginosa* (17). It has been suggested that the electromagnetic attraction of negatively charged bacteria to the positively charged Co-Cr oxide layer might be responsible for oxidation of the bacteria (24). Metal ions are suggested to inactivate proteins on the bacterial surface, decreasing membrane permeability, and eventually causing cell death (31). This would explain the lack of inhibitory effects in the metal ion groups.

Our XPS results showed a Mo-enriched particle surface composition. The surface oxide-film of Co-Cr alloy has been reported to consist of Co-Cr oxides, with Mo distributed in the inner layer. After dissolution in Hanks' solution, Co dissolves and the oxide layer consists of a Cr_2O_3 protective film layer (10) enriched by Mo (11). Recent work by Hart *et al.* (12) revealed that the metallic debris retrieved from tissues surrounding failed MOM hips contained Cr phosphate, metallic Co, but also oxidized Mo. Albeit limited literature is available on the bacteriostatic effects

of Mo, however, Yasasuki *et al.* recently presented data indicating that pure Mo is less toxic than pure Co towards *S. aureus* (IFO No 12732) using a novel film contact method (30). It is therefore not plausible that Mo is responsible for biofilm growth inhibition seen here.

In conclusion, Co-Cr particles possess bacteriostatic characteristics under dynamic growth conditions, unlike Co and Cr ions only.

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Chapter 5

Killing of staphylococcal biofilms on orthopaedic materials by gentamicin

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Submitted

Abstract

Background

Treatment of biomaterial-associated infection is complicated due to the resistance of biofilms to antimicrobials with a potential impact of the type of biomaterial involved. The aim of the present study was to compare the metabolic activity of three Staphylococcus epidermidis and two Staphylococcus aureus strains in biofilms grown on Ti, Co-Cr and ultra-high molecular weight polyethylene (UHMWPE) discs and relate the efficacy of gentamicin against these biofilms with metabolic activity.

Methods

Six hours old staphylococcal biofilms were grown in tryptone soya broth on Ti, Co-Cr and UHMWPE discs, after which growth was continued for another 18 h in the absence or presence of gentamicin (6 mg/mL). Biofilms were evaluated in terms of the number of adhering bacteria, their live/dead ratio and metabolic activity on the different biomaterials.

Results

The number of bacteria in the 6 h biofilms was similar on all biomaterials, with minor strain dependent differences. Metabolic activity per bacterium in 6 h old biofilms prior to growth was significantly lower in 3 out of the 5 strains in biofilms on hydrophobic UHMWPE as compared with more hydrophilic Ti and Co-Cr. The reduction of viable organisms after 18 h subsequent growth in the presence of gentamicin compared to biofilms grown in absence of gentamicin was lowest on UHMWPE, but no statistically significant correlation was found between lower metabolic active of 6h biofilms.

Conclusion

Orthopaedic biomaterials influence metabolic activity of staphylococcal biofilms, but metabolic activity did not correlate with staphylococcal killing by gentamicin in 24 h old biofilms.

Introduction

With the continuous increase in the use of biomaterials implants for the restoration of human function, also biomaterial-associated infections (BAI) occur with increasing numbers over the past decades. Reducing the incidence of BAI would provide a huge medical and financial benefit, as treatment is intensive and exhausts healthcare resources (4;20). *Staphylococcus epidermidis* and *Staphylococcus aureus* are the most frequently encountered strains in infected bone and joint samples (8;16) and are responsible for both chronic and acute BAI (7). When staphylococci adhere to a biomaterial implant, they form a biofilm. The otherwise relatively susceptible organisms form a slimy layer, which makes the bacteria more resistant to antibiotics and the host immune system (6;11).

In orthopaedics, the aminoglycoside gentamicin is the most frequently applied local antibiotic. It is one of the few heat-stable antibiotics suitable for use in acrylic bone cements (PMMA) for the treatment and prevention of BAI (10). Interestingly, the susceptibility of biofilms for gentamicin not only depends on the bacterial strain involved and the gentamicin concentration applied, but also on the material on which the biofilm is growing. On PMMA for instance, resistance of 24 h biofilms from a variety of clinically-derived and commercially-available staphylococci was larger than on stainless steel during 1 h exposure to gentamicin in concentrations up to 256 µg/mL (12). Slime production, as directed by the icaADBC operon, may be one of the mechanisms through which staphylococci protect themselves against antibiotics (1). Indeed, similar as with respect to the development of resistance against gentamicin of staphylococcal biofilms on different materials, also ica-expression and resulting slime-production seems to depend on the substratum involved. Slime production and gentamicin-resistance in three clinically-derived and one commercially–available, 24 h old *S. epidermidis* biofilms were higher on polyethylene than on stainless steel or PMMA when exposed during 24 h to gentamicin concentrations up 32 µg/mL gentamicin (14). Others have suggested that the biomaterial dictates antibiotic resistance without an influence of slime production (18). More in general, antibiotic resistance has been linked to the metabolic activity of surface adhering organisms (23) and for *Pseudomonas aeruginosa* it has been shown that low metabolic activity is correlated with a high tolerance to tobramycin and ciprofloxacin (9;22).

Since no such studies have been performed for staphylococcal biofilms on Co-Cr alloy, the aim of this study is to determine a possible correlation between antibiotic resistance and the metabolic activity of staphylococcal biofilms grown on cobalt-chromium alloy (Co-Cr) as compared with titanium alloy (Ti) and ultra-high molecular weight polyethylene (UHMWPE).

Materials and Methods

Bacterial strains

Two clinical isolates, *S. epidermidis* 64 (Sardjito Hospital, Yogyakarta, Indonesia (15)) and *S. aureus* 5298 (University Medical Center Groningen, The Netherlands (13)) and two ATCC strains *S. epidermidis* ATCC 12228 and *S. epidermidis* ATCC 35984 were used in this study. In addition, we included the bioluminescent *S. aureus* Xen36 (Xenogen, Alameda, CA). All strains were routinely cultured from frozen stock on blood agar plates at 37°C for 24 h in ambient air. A single colony was inoculated in 10 mL tryptone soya broth (TSB; OXOID, Basingstoke, UK) and incubated overnight at 37°C.

Minimal inhibitory concentrations (MIC) of gentamicin for all strains were determined using the E-test (Biomerieux, MarcyI`Etoile, France). To this end, a bacterial suspension with a density of 1.3 at 600 nm was streaked by a sterile cotton swab on a Mueller Hinton agar plate, and after air drying a gentamicin E-strip was added to the plate. Agar plates were incubated at 37°C for 24 h and the MIC was determined by reading the E-test. MIC values above 4 µg/mL gentamicin were considered indicative of resistance (2).

Substratum surfaces

Discs (0.2 cm thickness, diameter 1.0 cm, area 1.41 cm^2) of surgical-grade Ti (ISO 5832-3), Co-Cr (ISO 5832-12) and UHMWPE (gamma irradiated, not Vitamin E infused) were cut from a rod stock with a lathe and these were generously supplied by Biomet (Warsaw, IN). All discs were polished with a 6 and 3 µm diamond water-based suspension (Metadi, Buehler, Lake Bluff, USA) on a polishing cloth (Buehler, Lake Bluff, USA), cleaned by 5 min sonication in 2% alkaline cleaning agent (RBS 35, Omniclean, Breda, The Netherlands) followed by thorough rinsing with tap water,

sonication in ethanol and rinsing in ultrapure water. Ti and Co-Cr discs were passivated by sonicating the discs in 34% HNO_3 followed by thorough rinsing with ultrapure water. Subsequently, samples were glued to the bottom of a 12-wells plate (Costar, Corning, NY) with a polyurethane-rubber (Bison Kit Transparent, Bison Int., Goes, Belgium), previously evaluated not to affect bacterial viability and left to dry for 1 h.

Surface roughness of each of the samples were measured using atomic force microscopy (Veeco Instruments, Santa Barbara, CA) with a sharp SiN_3 tip in the contact mode. Water contact angles were measured using the sessile drop technique and a home-made contour monitor in order to determine the hydrophobicity of the different biomaterials surfaces. All physico-chemical surface characterization was done in triplicate with separate sample discs.

Biofilm formation, metabolic activity and gentamicin susceptibility

12-wells plates with the discs glued to the bottom of the wells were filled with 3 mL TSB and inoculated with 30 µL of an overnight culture of a staphylococcal strain. Discs were incubated for 6 h at 37°C after which the metabolic activities of the biofilms on the different biomaterials were determined by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; Sigma-Aldrich, Zwijndrecht, Netherlands) assay. MTT, a yellow soluble tetrazolium salt is reduced by metabolically active bacteria to a blue colored formazan (5). Briefly, 2 mL MTT solution (0.5 mg/mL) in PBS, containing 0.1% glucose and 10 µL menadion (10 µM), was added to each well. Plates were incubated at 37°C in the dark for 2 h. After removal of the MTT solution, biofilms were washed twice with demineralized water and resuspended in acid isopropanol (5% v/v 1 M HCl in isopropanol). Finally, the absorbance was measured at 560 nm using a spectrophotometer (Spectronic 20 Genesys™, Spectronic Instruments Inc., Rochester, NY).

In a separate set of experiments, 6 h biofilms formed on the different biomaterials in the 12-well plates were washed with phosphate buffered saline (PBS, 10 mM potassium phosphate and 150 mM sodium chloride, pH 7.0) and stained with *Bac*Light live/dead stain (Molecular Probes, Leiden, The Netherlands) and calcofluor white polysaccharide-binding stain (Optical Brightener, Sigma-Aldrich, Zwijndrecht, Netherlands). After 15 min in the dark, excess stain was

removed and biofilms were immersed in 3 mL PBS. Subsequently, confocal laser scanning microscopy (CLSM) images were collected using a Leica TCS SP2 with a 40x water objective. To prevent reflection by the discs at an excitation wavelength of 488 nm and emission filter settings of 600 nm and higher (red), sections were collected just above the surface, excluding imaging of the bacteria attached to the discs.

After imaging, discs were carefully removed with a pair of sterile forceps. Biofilms were detached by sonication on ice with a Vibra Cell 375 (Sonics and Materials, Danbury, CT). The resulting staphylococcal suspensions were subsequently diluted and bacterial concentrations were determined in a Bürker-Türk counting chamber. Numbers of staphylococci are presented per unit area. In order to assess bacterial viability, again live/dead stain was added to 10 µL of a staphylococcal suspension on a glass slide and live/dead ratios were determined with a fluorescence microscope.

Separately and in addition, wells with 6 h biofilms were refreshed with TSB (control) or TSB supplemented with 6 mg/mL bio-active gentamicin (Sigma-Aldrich, Zwijndrecht, Netherlands) and incubated for an additional 18 h. The gentamicin concentration was expected to represent local concentrations around gentamicin-loaded spacers and beads during initial burst release (17). Considering for instance, a volume of 10-15 ml joint fluid and an antibiotic release of 20 % in the first 24 h during local treatment of BAI with 3 strings of antibiotic-loaded beads containing 30 beads each with 4.5 mg active gentamicin per bead (17), a concentration of 6 mg/mL can be expected.

After 18 h of biofilm growth in the absence and presence of gentamicin, biofilms were characterized as described above and the percentage reduction of viable bacteria in biofilms exposed to gentamicin as compared to the control biofilm was calculated according to

$$\%\ \text{reduction} = \frac{\text{Viable count}_{\text{control}} - \text{Viable count}_{\text{gentamicin}}}{\text{Viable count}_{\text{control}}} * 100$$

Statistics

Differences in average counts, reductions and absorbance values were analyzed for significance by ANOVA and followed by a Mann-Whitney U-test. A 95% ($p < 0.05$, two-tailed) confidence interval was applied for statistical significance.

Results and Discussion

Four of the five strains involved were found to be susceptible for gentamicin with a MIC of less than 1 µg/mL (*S. epidermidis* ATCC 12228, *S. aureus* 5298, *S. epidermidis* 64 and *S. aureus* Xen36). *S. epidermidis* ATCC 35984 had a MIC of 32 µg/mL and was considered resistant to gentamicin. UHMWPE was found to be more hydrophobic (water contact angle 86 degrees) than Ti and Co-Cr surfaces (Table I), while the surface of Ti was 4 to 5 times smoother than Co-Cr and UHMWPE. All surface roughnesses however, were well below 1 µm. Co-Cr surfaces appeared to possess scratches, presumably as a result of machining the discs.

The total number of bacteria in 6 h old biofilms showed no differences between the biomaterials, while only small differences existed between strains (Figure 1A). The preference of *S. aureus* for adhesion to metal substrata, i.e. of *S. epidermidis* to adhere to polymeric substrata (3) could not be confirmed by our present results. Although surface roughness is also known to influence biofilm formation under conditions of fluctuating flow, most notably the oral cavity (19), this could not be confirmed under the static conditions of our experiments.

The viability of the 6 h biofilms was found higher than 95% in all strains. Metabolic activity expressed per bacterium in 6 h old biofilms, i.e. at the onset of the growth phase, was found to be significantly ($p < 0.05$) lower for all three *S. epidermidis* strains grown on UHMWPE as compared with their growth on Co-Cr (Figure 1B), while a similar tendency existed for *S. aureus* biofilms ($p > 0.05$). Moreover, a tendency of lower metabolic activity on UHMWPE compared with Ti was revealed, but this was only significant ($p < 0.05$) for *S. epidermidis* ATCC 12228. The 24 h old biofilms contained more bacteria than 6 h old ones, although the data suffer from quite large standard deviations (Figure 2). There is a tendency for all strains toward less biofilm formation on UHMWPE than on the metal alloys, but this was only significant ($p < 0.05$) for biofilms of *S. epidermidis* ATCC 35984 on Co-

Cr. The viability of all 24 h old biofilms is high and varies on average from 97% for UHMWPE to 89% for Co-Cr. Biofilms exposed to gentamicin showed significantly ($p < 0.05$) lower total bacterial counts and lower viabilities than biofilms not exposed to gentamicin (see also Figure 2). Gentamicin was particularly effective against *S. aureus* Xen36, which might be due to the low number of *S. aureus* Xen36 in the control biofilm.

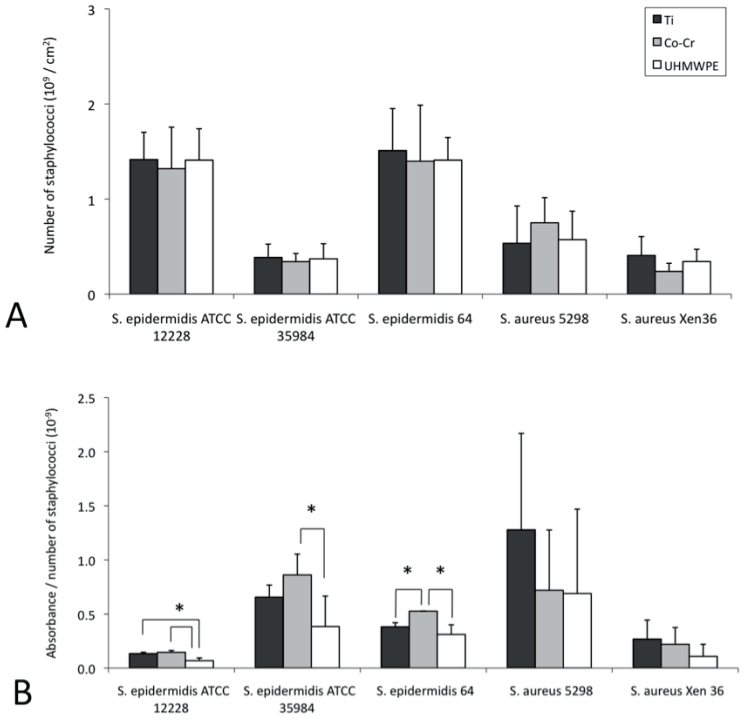
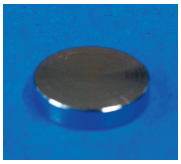
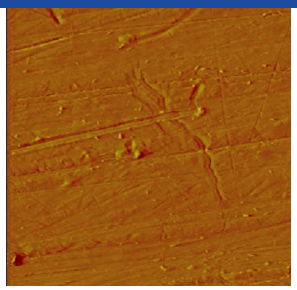
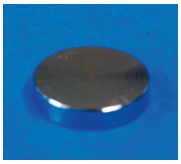
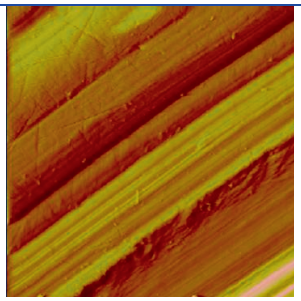
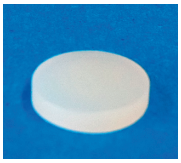
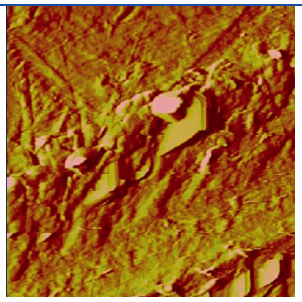


Figure 1. (A) The number of staphylococci per unit area in 6 h old biofilms formed on Ti, Co-Cr and UHMWPE discs. The viability 6 h biofilms was found to be higher than 95% in all strains (not shown in figure). (B) Metabolic activities by an MTT assay expressed as absorbance per bacterium in 6 h biofilms on Ti, Co-Cr and UHMWPE discs for the different staphylococcal strains employed in this study. Data represent mean ± standard deviations over three experiments with separately cultured bacteria. *indicates a significant difference in the metabolic activity of bacteria grown on Ti, Co-Cr or UHMWPE discs.

Table I. Sample discs of the different orthopaedic materials used, together with AFM images of their surfaces and their surface roughnesses and water contact angles. Data represent averages with ± indicating the SD over 3 separate experiments.

	AFM image (5 x 5 µm)	Roughness (nm)	Contact angle (degrees)
Ti 		85 ± 46	45 ± 0
Co-Cr 		401 ± 218	45 ± 5
UHMWPE 		294 ± 66	86 ± 4

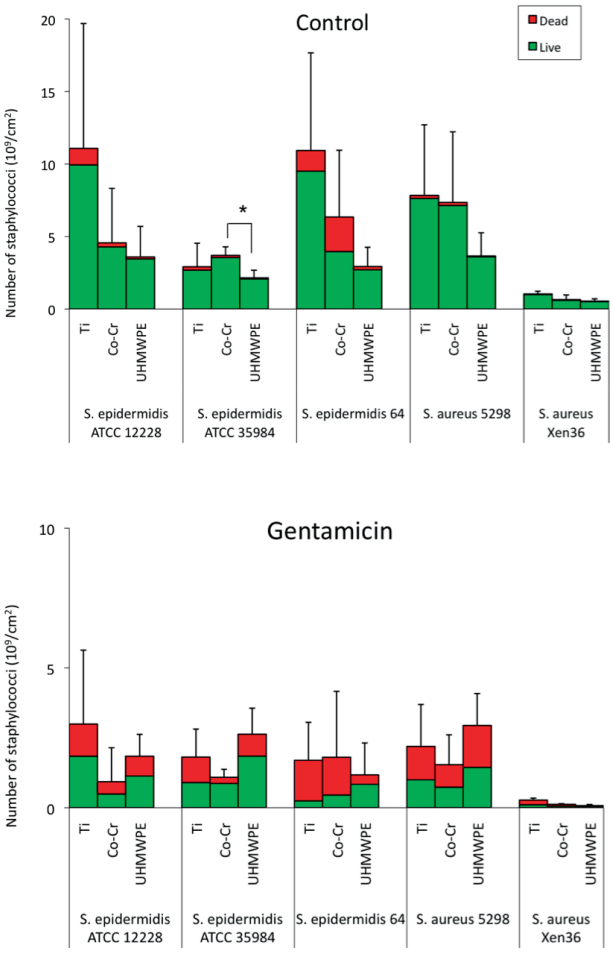


Figure 2. The number of live (green) and dead (red) staphylococci per unit area as derived from 24 h old biofilms formed on Ti, Co-Cr and UHMWPE discs prior to (top) and after exposure to gentamicin (bottom). Data represent mean ± standard deviations over three experiments with separately cultured bacteria. *indicates a significant difference in the number of bacteria grown on Co-Cr and UHMWPE.

The numbers of viable Staphylococci as presented in Figure 2 have been used to calculate a percentage reduction in the number of viable organisms on the different biomaterials upon treatment by gentamicin. Figure 3 shows that staphylococcal killing on UHMWPE is generally less than on the metal alloys, although this was only significant for *S. epidermidis* ATCC 35984 ($p < 0.05$). Possibly, the use of a high concentration of gentamicin (6 mg/mL) overrules all effects of differences in substratum properties. *In vivo*, drainage fluid of patients with concentrations as high as only 440 µg/mL gentamicin have been reported, which does not rule out however, that much higher local concentrations may develop around antibiotic-loaded beads and spacers (21). Interestingly, despite the use of relatively high gentamicin concentrations, killing efficacy never reached 100%.

CLSM analysis of biofilms from all strains grown in the presence of gentamicin showed an increased slime production on all substrata with respect to biofilms grown in the absence of gentamicin (see Figure 4). Nuryastuti et al. observed more extensive slime-production on UHMWPE as compared with stainless steel and PMMA, but gentamicin concentration used were much lower (16-32 µg/mL) than applied here, which might explain the massive slime-production observed regardless of the substratum properties (14).

In conclusion, orthopaedic biomaterials influence metabolic activity of staphylococcal biofilms, but metabolic activity did not correlate with staphylococcal killing by gentamicin in 24 h old biofilms. The reduction of viable organisms grown in the presence of gentamicin compared to biofilms grown in absence of gentamicin was lowest on UHMWPE, which was the most hydrophobic biomaterial involved in this study. However, no statistically significant correlation was found between lower metabolic activity at the onset of growth and lower staphylococcal killing by gentamicin.

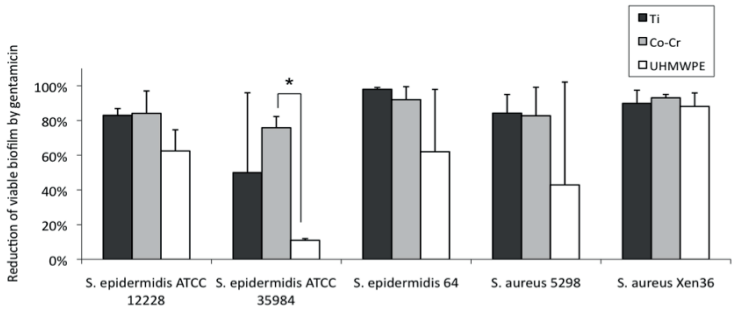


Figure 3. Percentage reduction in the number of viable staphylococci in 24 h old biofilms formed Ti, Co-Cr and UHMWPE discs upon exposure to gentamicin as compared to untreated 24 h control biofilms. Killing efficacy represents mean ± standard deviations over three experiments with separately cultured bacteria. *indicates a significant difference in staphylococcal numbers reduction of bacteria exposed to gentamicin compared to the control.

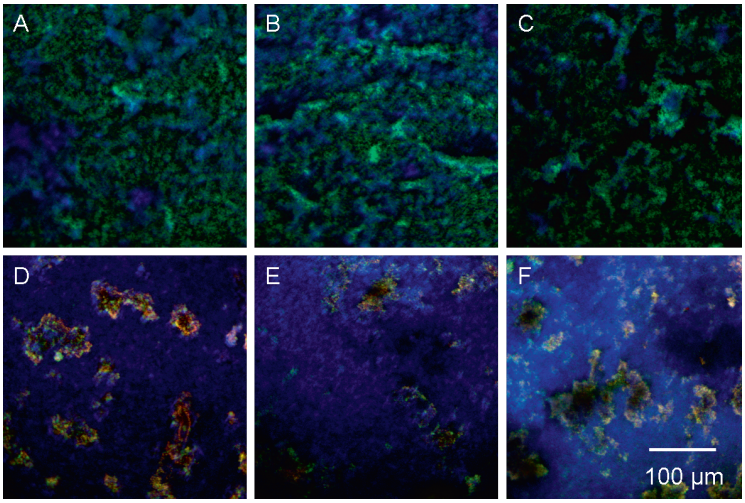


Figure 4. CLSM images (375 x 375 µm) of 24 h old *S. epidermidis* ATCC 35984 biofilm grown on Ti, Co-Cr and UHMWPE discs prior to (A, B and C) and after exposure to gentamicin (D, E and F). *Bac*light staining visualizes live bacteria (green) and dead bacteria (red). Slime (blue) is visualized by calcofluor white. Colors are based on the average voxel intensity throughout the depth of the biofilm.

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Chapter 6

The influence of Co-Cr and UHMWPE particles on infection risk: An *in vivo* study in mice

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Abstract

Background

Wear of metal-on-metal (cobalt-chromium, Co-Cr particles) and metal-on-polyethylene (ultra-high-molecular-weight polyethylene, UHMWPE particles) bearing surfaces in hip prostheses is a major problem in orthopedics, suggested to cause inflammatory reactions and a-septic loosening of prostheses. However, the influence of wear particles on the risk of infection is still unclear. This study aimed to compare the influence of Co-Cr and UHMWPE particles on the risk of infection.

Methods

Bioluminescent *Staphylococcus aureus* Xen36 were injected in air pouches prepared in subcutaneous tissue of immuno-competent BALB/c mice (control), as a model for the joint space, in the absence or presence of Co-Cr or UHMWPE particles. Bioluminescence was monitored longitudinally up to 21 days, corrected for absorption and reflection by the particles and expressed relative to the bioluminescence found in the presence of staphylococci only. After termination, air pouch fluid and air pouch membrane were cultured and histologically analyzed.

Results

Bioluminescence was initially lower in mice exposed to UHMWPE particles with staphylococci than in mice injected with staphylococci only, possibly because UHMWPE particles initially stimulated a higher macrophage presence in murine air pouch membranes. For mice exposed to Co-Cr particles with staphylococci, bioluminescence was observed to be higher in two out of six animals compared to the presence of staphylococci alone. Accordingly, a high number of macrophages was found in the air pouch of the mouse showing the most prolonged, elevated bioluminescence.

Conclusion

In the majority of mice, infection risk in the absence or presence of Co-Cr and UHMWPE particles appeared similar, assuming that the longevity of an elevated bioluminescence is indicative of a higher infection risk. However, the presence of Co-Cr particles yielded a higher bioluminescence in two out of six mice, possibly because the macrophage degradative function was hampered by the presence of Co-Cr particles.

Introduction

Metal-on-metal (MOM) bearings have been favored compared to conventional metal-on-polyethylene (MOP) bearings, because of their low wear in hip arthroplasties for young and active patients (7;24). However, with up to three million gait cycles (20), active MOM patients still accumulate nearly 1 mg of nano-sized cobalt-chromium (Co-Cr) wear particles per year (1). Patients with MOP bearings gather approximately 20- to 100-fold more ultra-high-molecular-weight polyethylene (UHMWPE) particles after the same number of cycles (22).

Conflicting results have been reported concerning the influence of Co-Cr wear particles on bacterial growth and associated infection risks of total hip arthroplasties with MOM-bearings. Co-Cr debris has been demonstrated to promote bacterial growth *in vitro* (2), but on the other hand high concentrations of Co and Cr ions appeared to possess bacteriostatic properties as well (14). Recently, Co ions were reported to inhibit neutrophil proton pumps (6) and Co-Cr particles induced toxic effects when phagocytosed (15;26), frustrating the degradative function of macrophages. These processes hamper the immune system and might predispose MOM patients to infection at the implant site (27-29).

In vitro it is impossible to determine the net effect of wear particles in the presence of bacteria on infection risks. Clinically, investigating the influence of wear particles on the development of an infection requires large patient groups as infection only occurs in 0.5 - 3% of patients (5). Moreover, it is not feasible to collect pseudosynovial fluid for infection analysis from such a large patient group, as this invasive procedure bears the risk of introducing an infection.

An *in vivo* murine air pouch model has been developed in order to mimic the joint lining around failed prostheses for comparing the biocompatibility of particulate debris. Using this model, the presence of Co-Cr, UHMWPE, Ti-6Al-4V and polymetylmethacrylate (PMMA) particles in such pouches were found to increase the air pouch membrane thickness, and the number of macrophages in the pouches as well as the IL-1 response (25). Forty-eight hours after the introduction of particles into such pouches, Ti-6Al-4V showed the most pronounced increase in membrane thickness and IL-1 response, while UHMWPE particles were responsible for the highest increase in number of macrophages. In addition, both shape and texture of the particles were found to influence the

severity of inflammatory response, with rough debris surface texture exerting the most adverse tissue response (21). The effects of the combined presence of bacteria and particulate debris in an air pouch model of the joint lining around a prosthesis has never been performed.

In the current manuscript, we aim to compare the influence of Co-Cr and UHMPE particles on the risk of infection in an animal model. To this end, an *in vivo* bio-optical imaging system was used to longitudinally monitor the growth of bioluminescent staphylococci in air pouches prepared in the subcutaneous tissue of mice in the absence and presence of Co-Cr or UHMWP particles. After sacrifice, microbiological and histological analyses of surrounding tissue were performed.

Methods

Wear particles

Commercially available pure UHMWPE particles (Ceridust 3615, 7 µm mean diameter) were generously provided by the manufacturer (Clariant, Coventry, RI). Co-Cr particles were derived from ISO 5832-4 cast Co-Cr alloy Micro-Melt® dust (Carpenter Powder Products, Wyomissing, PA), with courtesy of Biomet (Warsaw, IN.). In order to obtain Co-Cr particles with a size range similar to UHMWPE particles, we used a pre-separator attached to an inhaler 2000 adapter (Sympatec GmbH, Clausthal-Zellerfeld, Germany) to remove larger particles. Subsequently, the smaller particles were trapped in an aerosol and liquid impinger in ethanol. Both Co-Cr and UHMWPE particles were washed in 70% ethanol solution to remove possible bound endotoxin from environmental bacteria and ethanol was subsequently evaporated under vacuum. The absence of endotoxins was confirmed using the Limulus assay (Endosafe, Charlestown, SC). Prior to inoculation, particles were sonicated for 30 min to prevent aggregation. Final particle size distributions were measured with a Sympatec HELOS compact KA laser diffraction apparatus (Sympatec GmbH, Clausthal-Zellerfeld, Germany), using a RODOS dry powder disperser at 3.0 bar. A lens of 200 mm was used and calculations were based on the Fraunhofer diffraction theory. Particle morphology was visualized with a JEOL JSM6301 scanning microscope (JEOL, USA Inc., Peabody, MA, USA) at 3 kV, after sputter-coating the particles with a 5 nm thick, conductive film of Pd/Au.

Bioluminescent Staphylococcus aureus Xen36

S. aureus Xen36 (commercially obtained from Xenogen, Alameda, CA, USA) was derived from *S. aureus* ATCC 49525. The parental strain was a clinical isolate, made bioluminescent by stably integrating a modified lux operon *(Photorhabdus luminescens luxABCDE)* on a native plasmid. This modification enables the strain to produce luciferase and its substrate, resulting in a photon-emitting state when in presence of ATP, the reduced biomolecule flavin mononucleotide, NADPH and oxygen (12).

S. aureus Xen36 was cultured from cryopreservative beads (Protect Technical Surface Consultants Ltd, Lancashire, UK) onto tryptone soya broth (TSB; Oxoid, Basingstoke, UK) agar plates supplemented with 200 µg/mL kanamycin at 37°C in ambient air. One colony was used to inoculate 10 mL TSB supplemented with kanamycin for 24 h at 37°C. Main cultures were grown overnight by inoculating 10 mL TSB supplemented with kanamycin with 0.5 mL preculture. Subsequently, main cultures were centrifuged and rinsed three times with sterile phosphate-buffered saline (PBS) and resuspended in PBS. The inoculum was diluted to 9×10^8 cells/mL by counting in a Bürker-Türk counting chamber. Aliquots of the inoculum were incubated on agar plates for 48 h at 37°C to yield the concentration of CFUs in the inoculum, which was found to be 5×10^8 CFU/mL.

For animal experiments, a 5% weight/volume (25) concentration was chosen for Co-Cr particles (25 mg/mL) as suspended in syringes with 1 mL of the staphylococcal suspension. The concentration of UHMWPE particles was adjusted to match the number of Co-Cr particles using the particle size distributions listed in Table I, resulting in a concentration of 5 mg/mL. Although the bioluminescence yield per bacterium should not *a priori* be influenced by its environment, the presence of light reflecting or absorbing surfaces like Co-Cr or UHMWPE particles may influence the bioluminescence captured in bio-optical imaging. Therefore, syringes filled with only *S. aureus* Xen36 and with Co-Cr or UHMWPE particles added were imaged with the bio-optical imaging system, as described below. The ratio between the bioluminescence yields in the absence and presence of Co-Cr or UHMWPE particles was used to correct the *in vivo* bioluminescence for possible effects of absorption and reflection by the particles, assuming that the influence of

differences in geometry between the syringes and the air pouches can be neglected.

Table I. Cumulative volume distributions of Co-Cr and UHMWPE particles, measured with laser diffraction.

Cumulative distribution (%)	Co-Cr	UHMWPE
	Particle size beneath (μm)	
10	4.8	3.7
25	6.5	5.8
50	9.4	8.3
75	16.1	11.2
90	24.3	14.1
99	35.6	18.8

Murine air pouch infection model

Seven weeks old male BALB/c OlaHsd immune competent mice (Harlan Netherlands BV, Horst, Netherlands) with specified pathogen free conditions were quarantined in our animal facility for two weeks prior to experiments. All animals were housed per groups of three in individual ventilated cages with Cellu-dri Soft R bedding (Shepherd Speciality papers, Kalamazoo, Michigan, US). The mice were weighed daily and evaluated on the basis of activity, illness and alternative behavior. All mice weighed 20 g or over at the start of the experiment (average 22.2 ± 1.0 g) and had *ad libitum* access to food and water throughout the experiment.

Air pouches were prepared through subcutaneous injections of sterile air (10). Briefly, an area of 2 cm^2 dorsal skin was shaved and cleaned with alcohol to provide the pouch site. Sterile air (1 mL) was injected subcutaneously at a single site with a 25-gauge needled syringe on alternate days for 5 days to establish a definitive pouch, which filled spontaneously with serum. Buprenorphine (0.03 mg/kg) was administered subcutaneously 30 min prior to injections. During pouch preparation, anesthesia was induced with a 3.5% Isoflurane/O2 (Zeneca, Zoetermeer, Netherlands) gas mixture and maintained at 1.5%.

6 days after air pouch creation, pouches were injected with 1 mL of a bioluminescent staphylococcal suspension (6 mice) or a combination of bioluminescent bacteria with Co-Cr or UHMWPE particles (also 6 mice per group). In addition, three smaller control groups (3 mice per group) were injected with Co-

Cr or UHMWPE particles or only PBS in the absence of any bacteria. Subsequently, bacterial presence was monitored for a period of 21 days by bioluminescence imaging, as described below. At the end of the experimental period, animals were euthanized under anesthesia by cervical dislocation.

Experiments were performed in accordance to federal legislation regarding the protection of animals and were approved by the Animal Experiments Committee at the University of Groningen.

Bioluminescence imaging

Bioluminescence was imaged with a highly-sensitive, cooled CCD camera (IVIS 100 Imaging System, Caliper Life Sciences, Hopkinton, MA, USA). Bioluminescence images were obtained with 5 min exposure time using a 15-cm field of view, binning of 4, 1/f stop and open filters. Pseudo-color images were obtained and overlaid on a gray scale photograph of the mice. Mice were kept under anesthesia during imaging. Bioluminescence was quantified as total bioluminescent flux (photons/s) within a circular region of interest (2.5 cm^2) by using living image software (Xenogen, Alameda, CA, USA). In case of imaging of the syringes filled with bioluminescent bacteria in the absence and presence of particles, the area of the syringe was taken as the region of interest.

In order to account for effects of the presence of particles on staphylococcal bioluminescent fluxes measured, all measured fluxes were corrected for absorption and reflection using the *in vitro* bioluminescence ratios between syringes filled with bacteria in the absence and presence of particles. Subsequently, data of the groups of mice with *S. aureus* Xen36 in combination with particles were normalized by dividing the corrected bioluminescence flux of individual mice on each day by the average corrected bioluminescence flux observed in the presence of *S. aureus* Xen36 only.

Analysis of tissue samples

Air pouch fluid was collected during anesthesia by flushing an air pouch with 1 mL sterile PBS, immediately before euthanizing the mice. Directly after euthanasia, air pouch membrane samples were collected from the dorsum of the air pouch for histological and culturing purposes.

For histological analysis, tissue samples were taken from all animals. Part of a tissue sample was fixed in 10% buffered formalin, dehydrated, and embedded in paraffin blocks with particular care to preserve the original shape of the pouch tissue, while the other part was used for culturing. Sections were cut along the pouch middle line, mounted and stained with hematoxylin and eosin stain. Cover slips were fitted to the slides using glass bonding adhesive.

After scanning with a NanoZoomer 2.0-HT system (Hamamatsu photonics, Hamamatsu, Japan), three separate sections per specimen were evaluated with NDP view software (Olympus, Center valley, PA, USA) in a blinded fashion by three individuals. Tissue reaction was scored by measuring the thickness of the pouch membrane and enumeration of the number of macrophages per mm^2 of analyzed air pouch membrane.

For culturing, tissue samples were sonicated on ice with a Vibra Cell 375 (Sonics and Materials, Danbury, CT) and subsequently rubbed with sterile forceps on TSB agar plates. Both the sonication fluid from tissue samples and the air pouch fluid were serially diluted and spread on TSB agar plates. All plates were left to incubate for 24, 48 and 72 h at 37°C and CFUs were counted.

Statistics

Normalized bioluminescence fluxes were compared at each observation day during the 21-days follow-up period by ANOVA followed by a Mann-Whitney U-test. In addition, air pouch membrane thickness and macrophage counts were analyzed by ANOVA followed by a Mann-Whitney U-test. P values of < 0.05 were considered significant.

Results

Laser diffraction revealed volume size-distributions to be within the micrometer size range (Table I). Differences between Co-Cr and UHMWPE particles were minor and confined to particle sizes above 10 µm. SEM images demonstrated that Co-Cr particles are more spherically shaped, with UHMWPE particles having a more irregular flake-shape (Figure 1).

The bioluminescence captured in the bio-optical imaging system was influenced by the presence of Co-Cr and UHMWPE particles, as can be seen in Figure 2. Bioluminescence arising from 1 mL syringes filled with 5 x 10^8 *S. aureus* Xen36 in the presence of Co-Cr particles was significantly weaker (0.8x) than in the absence of Co-Cr particles, due to absorption of light by the particles. On the other hand, extensive reflection by UHMWPE particles significantly increased the bioluminescence measured (1.6x). These ratios were employed to correct the bioluminescence arising from mice infected with *S. aureus* Xen36 in the presence of particles.

Figure 3 shows examples of bioluminescence images of an individual mouse from each of the three experimental groups, followed longitudinally. Weakest bioluminescence is seen for the mouse injected with *S. aureus* Xen36 and Co-Cr particles, while more prominent and lasting bioluminescence arises from the mouse injected with *S. aureus* Xen36 and UHMWPE particles. These images however, may not be taken as indicative for the course of infection, as they do not account for absorption and reflection of the bioluminescence signal.

Bioluminescence from all three groups of mice, as corrected for absorption and reflection were subsequently normalized by dividing the corrected bioluminescence fluxes by the average bioluminescence flux at each day in the group of mice injected with bacteria only. Figure 4 reveals a clear difference in the course of infection in animals having received Co-Cr particles and UHMWPE particles. In the Co-Cr group, normalized bioluminescence was below unity in four out of the six mice. In the two mice with bioluminescence above unity, bioluminescence returned to unity within 9 to 21 days. In the UHMWPE group, bioluminescence was initially less than unity, with only one animal on day 3 emitting a slightly higher bioluminescence, but after 9 days there was no difference anymore in bioluminescence arising from mice injected with *S. aureus* Xen36 only or with staphylococci and UHMWPE particles.

Background bioluminescence flux was found to be below 1.2 x 10^5 (p/s) in control groups injected with PBS or particles only and coincided with the bioluminescence observed in all groups of mice after 21 days. Histological analyses revealed no signs of inflammation in 26 out of the 27 mice in the different groups after sacrifice at day 21 (Figure 5).

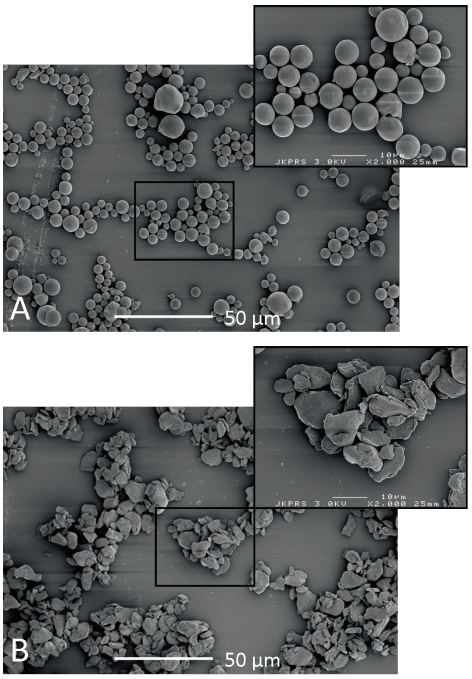

Figure 1. Scanning electron micrographs of Co-Cr (**A**) and UHMPWE (**B**) particles, showing clear differences in morphology between both particles.

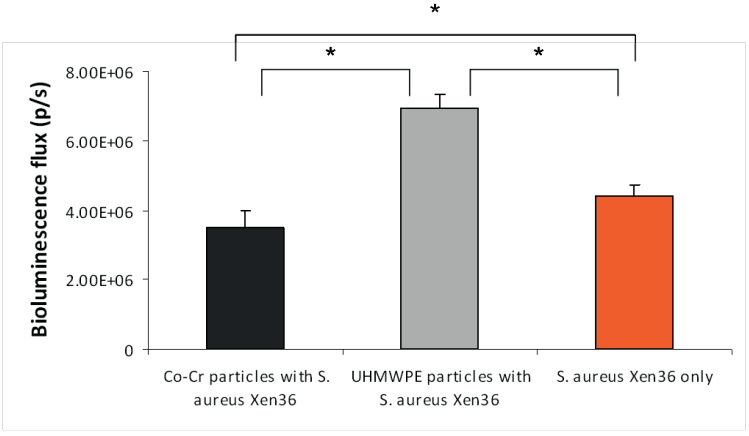

Figure 2. Bioluminescence flux from 1 mL syringes filled with 5 x 10^8 CFUs *S. aureus* Xen36, with or without 5 mg Co-Cr or 25 mg UHMWPE particles added. * indicates a significant difference in bioluminescence flux ($p < 0.05$).

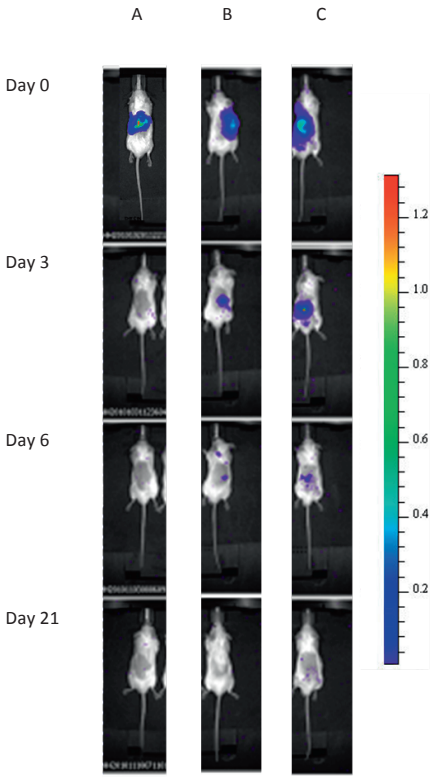

Figure 3. Bioluminescence images of mice injected with *S. aureus* Xen36 alone (C) and with Co-Cr (A) or UHMWPE (B) particles added, taken immediately after injection (day 0) and 3, 6 and 21 days after injection. The scalebar represents bioluminescence flux (x 10^6 p/s/cm^2/sr). Note that images do not account for absorption and reflection of the bioluminescence signal.

Average air pouch membrane thickness after sacrifice was found to be 1.3 ± 0.6 mm with no significant differences across groups (Table II). Similarly, mice injected with staphylococci in the absence or presence of particles, including control mice, had no significant difference in macrophage counts after sacrifice, with the exception of the animal in the Co-Cr and *S. aureus* Xen36 group for which a high bioluminescent signal was measured until day 18. This mouse showed a high relative macrophage count of 12.4. In addition, this mouse was observed to possess a post-inflammatory hematoma. Particulate debris was never detected in any tissue, while extensive culturing did not reveal *S. aureus* Xen36 bacteria in any of the tissue samples.

Table II. Relative air pouch membrane thickness and numbers of macrophages contained in the presence of Co-Cr or UHMWPE particles, expressed relative to the membrane thickness and numbers of macrophages in the presence of *S. aureus* Xen36 only (1.6 ± 0.6 mm and 89 ± 32 macrophages/mm^2).

	Number of animals	Relative membrane thickness	Relative number of macrophages
S. aureus Xen36 only	6	1	1
PBS only	3	0.8	1.4
Co-Cr particles only	3	0.7	1.4
UHMWPE particles only	3	0.7	1.8
UHMWPE particles with *S. aureus* Xen36	6	0.9	1.5
Co-Cr particles with *S. aureus* Xen36	6	0.7	1.4*

All data were obtained after sacrifice at 21 days post-particle injection.
* indicates data over five animals excluding, the animal showing consistent high bioluminescence fluxes until day 18, for which the relative macrophage count was 12.4 at sacrifice.

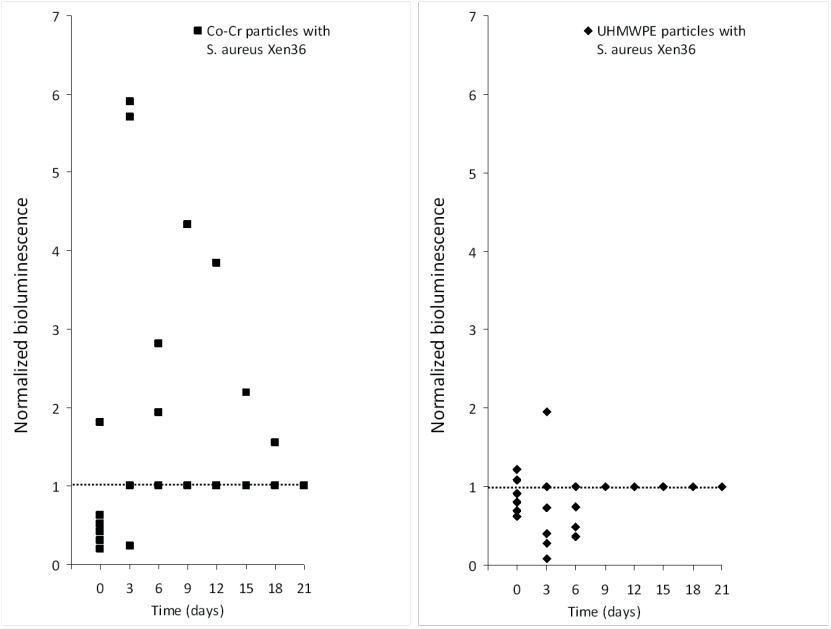

Figure 4. Normalized bioluminescence flux as a function of time after correction for absorption and reflection due to the presence of Co-Cr or UHMWPE particles. The normalized bioluminescence flux denotes the flux arising from air pouches filled with particles and bacteria relative to the bioluminescence arising from pouches filled with *S. aureus* Xen36 only. Each symbol represents one mouse.

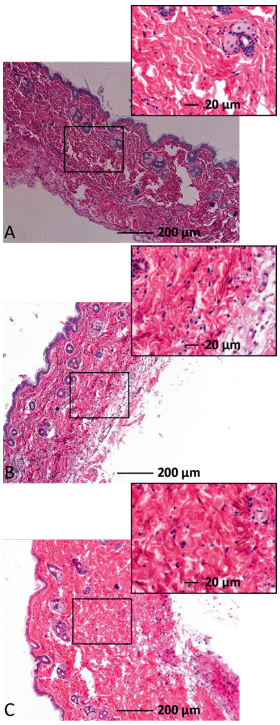

Figure 5. Air pouch membranes after hematoxylin and eosin staining of three mice exposed to Co-Cr particles and *S. aureus* Xen36 (**a**), UHMWPE particles and *S. aureus* Xen36 (**b**) and *S. aureus* Xen36 only (**c**) showing no signs of infection or inflammation due to particles or bacteria.

Discussion

Long-term clinical data on infection rates for specific bearing types are not yet available. Although there are *in vivo* studies available on the influence of bulk material on the incidence of infection (19), this is the first *in vivo* study towards assessing the influence of wear particles on bacterial infection risk. In this study, we used a novel method of *in vivo* imaging to longitudinally monitor the persistence of bioluminescent staphylococci in an artificial joint space. In the majority of mice, infection risk in the absence or presence of Co-Cr and UHMWPE particles appeared similar, assuming that the longevity of an elevated bioluminescence is indicative of a higher infection risk. However, the bioluminescence signal was initially lower in

mice exposed to UHMWPE particles with staphylococci than in mice injected with staphylococci only, possibly because UHMWPE particles initially stimulated a higher macrophage presence in murine air pouch membranes (21;25). For mice exposed to Co-Cr particles with staphylococci, bioluminescence was observed to be higher in two out of six animals compared to the presence of staphylococci alone and accordingly a high number of macrophages was found in the air pouch of the mouse showing the most prolonged, elevated bioluminescence. Therefore it can be concluded that whereas the presence of UHMWPE particles does not increase the risk of infection, Co-Cr particles may have variable effects on infection risk in different individuals. This study can not provide a clue as to why two mice injected with Co-Cr particles and *S. aureus* showed elevated bioluminescence for prolonged periods of time as compared to the four other mice in this group, showing a response similar to the one of mice injected with UHMWPE particles and *S. aureus*.

Although an increased number of macrophages was reported 48 h after injection of Co-Cr or UHMWPE particles into murine air pouches (25), it is clear from the present study that macrophage numbers reduce to control levels within 21 days even during simultaneous exposure to *S. aureus* Xen36. If, for unknown reasons, a relatively fulminant infection occurs, higher numbers of macrophages persist for longer periods of time. This occurred in two mice of the Co-Cr group, which may not be a coincidence since it has been shown that macrophages in the presence of Co-Cr particles with staphylococci can be prone to toxic effects of Co ions (6;15;26), frustrating their degradative function. This evidently does not necessarily occur in all animals. Generally, histological studies of tissues retrieved from revised prostheses have shown infiltration of surrounding tissues with Co-Cr or UHMWPE debris (8). In our study, particulate debris was not visible in any histological sample, nor was there any sign of inflammation after 21 days observed in five out of six animals injected with Co-Cr particles and *S. aureus* Xen36. This may imply transport of particles to other parts of the body, as described earlier in patients where metal wear debris has been found in lymph nodes, liver and spleen (23), despite the fact only 10 % of our particles were sized beneath 4 µm. Clinically derived particles from periprosthetic tissues size between 40-120 nm for Co-Cr (9;17) and are around 660 nm for UHMWPE (18). However, more than 50 % of the

particles used were in the phagocytosable size range smaller than 10 micrometer (13) and may be expected to provoke inflammatory reactions *in vivo* (30).

One of the benefits of using bioluminescence is the relative ease with which bacterial presence can be monitored longitudinally in one and the same animal (3). This is the first study to combine the use of an *in vivo* air pouch model with bioluminescence measurements to evaluate bacterial persistence in the absence and presence of wear debris. All current studies employing bioluminescence measurements to assess the longevity and severity of biomaterials-associated infections have used subcutaneous implantation of biomaterials. Kadurugamuwa *et al.* pre-inoculated a Teflon catheter and measured significant *in vivo* bioluminescence signals up to 20 days (16). Engelsman *et al*. pre-inoculated surgical meshes of different composition and found higher levels of bioluminescence up to 10 days that coincided with *ex vivo* counts of the number of colony forming units that could be retrieved from explanted meshes (11). The detection limit of bioluminescence measurements to detect bacterial presence is much lower than of, for instance, plate counting, and bacterial persistence in tissues surrounding infected biomaterial has been demonstrated despite the absence of measurable bioluminescence (12). In fact, it has been suggested that bacteria on and around an implanted biomaterial not only find protection against antibiotics and the host immune system in their biofilm mode of growth, but also seek protection in surrounding tissue (4). It might well be a unique feature of this air pouch model that it does not allow bacteria to migrate and find shelter in surrounding tissue, possibly because the air pouch membrane is heavily vascularized.

In conclusion, the presence of staphylococci in combination with UHMWPE particles did not increase the risk of infection, possibly as a result of initially higher macrophage levels. The combination of staphylococci with Co-Cr particles is known to frustrate the degradative function of macrophages and accordingly bioluminescence was higher and more prolonged in two out of six mice, suggesting that Co-Cr particles may increase the risk of infection. However, influences of the presence of particulate material in combination with staphylococci, had disappeared after 21 days, regardless of the type of particles.

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Chapter 7

Higher revision risk in metal-on-metal bearings compared to metal-on-polyethylene bearings: An Australian National Joint Replacement Registry review of 100,906 hip arthroplasties

Abstract

Background

There is an ongoing need to review large series of hip arthroplasties performed with use of metal-on-metal (MOM) and metal-on-polyethylene (MOP) implants. We now evaluate such a nationwide series for the influence of bearing type on the revision risk.

Methods

Records of hip arthroplasties, performed between September 1999 and December 2008, were retrieved from the Australian National Joint Registry and retrospectively reviewed at a mean of 3.4 years after the primary procedure. Comparisons were made between age, gender, indications for the index procedure and head size of the prosthesis between patients with MOM and MOP implants using Pearson's chi-square test. Cox proportional-hazard models were performed to determine predictors of revision for infection. Cumulative curves were used to describe the infection- and non-infection revision risk.

Results

100,906 hip arthroplasties, of which 17,510 MOM and 83,396 MOP implants, were performed during our study period. 2720 prostheses were revised (2.7% of total). The overall cumulative 8-year revision rate was 5%; 6% for MOM implants and 5% for MOP prostheses. The cumulative 8-year revision rate for infection was 1.2% for MOM implants and 0.8% for MOP prostheses. MOM bearings with a head size > 32 mm had a hazard ratio for revision of 1.24 compared to MOP prostheses with a head size ≤ 32 mm. In addition, MOM bearings with a head size > 32 mm were found to be revised more often for infection (0.3 revisions per 100 observed years) compared to prostheses with a smaller head size (0.1 revisions per 100 observed years).

Conclusion

The Australian National Joint Registry revealed significantly higher revision rates of MOM implants, specifically with a head size > 32 mm compared to MOP prostheses.

Introduction

Worldwide there is an increasing number of total hip revisions (9). The accompanying high costs and increased patient morbidity of revision surgery are reasons for the scientific society to review these failures and their possible causes. Since the introduction of joint registries (18;20;34), there has been the possibility of comparing survival rates of nationwide numbers of prosthetic implants. However, no comparison on bearing types has been performed yet.

Metal-on-metal (MOM) hip implants have been extensively implanted throughout many institutions worldwide from the time they were re-introduced, at the end of the last century (8;10). Due to the hypothesis that wear products of the established metal-on-polyethylene (MOP) were responsible for osteolysis (41), development of new bearings was at that time endorsed by the orthopaedic society. Remarkably low wear of MOM bearings in large diameter implants has led to its resurgence in use in young and active patients (13;32;38-40). However, MOM implants are not immune to osteolysis (31). In addition, metal alloys used in MOM bearings degrade by wear, corrosion, or a combination of the two (26;42) and the clinical impact of these particles is for the most part unclear. Extensive research efforts have been done to determine the consequences of wear particles and its corrosion products on osteolysis or other reasons for revision (11;16;23). Although recent results towards the use of MOM implants are encouraging in several small and a few larger clinical series (3;4;30), there is the need for evaluation of larger numbers, such as can be obtained from a Joint Registry.

Approximately 9-15% of all revisions are carried out for infection (1;35), indicating the importance of this postoperative complication. Infection risk might be influenced by wear particles as planktonic bacterial growth is stimulated more by MOM particles than by MOP wear debris (6). On the other hand, high concentrations of metal ions derived from salts have shown to possess bacteriostatic effects (22). There is limited literature available on the clinical impact of these findings, as long-term results on revision rates and especially infection rates are scarce in newly introduced hip arthroplasties.

The purposes of this study were, firstly, to report on the early results of a nationwide series of hip arthroplasties performed with MOM and MOP implant designs, and secondly, to evaluate the effect of diverse demographic and clinical

variables on revision rate and infection rate in particular, with regard to gender, age, the indications for the index procedure, bearing type and head size of the prosthesis.

Material and methods

Source of data

The Australian Orthopaedic Association National Joint Replacement Registry (AOANJRR) was established in 1999 and data collection became fully national during 2002 (18). It stores information on primary and revision joint arthroplasties performed in Australia.

Between 1 September 1999 and 31 December 2008, all recorded MOM and MOP implants were included. Subsequently, the number of recorded revisions was extracted, as defined in the Joint Registry as exchange or removal of one or more components. We analyzed the effect of age, gender, the indications for the index procedure (i.e. primary osteoarthritis, rheumatoid arthritis or other, not further specified), bearing type (i.e. MOM versus MOP) and head size (> 32 mm or ≤ 32 mm) on revision rate. Primary conventional hip arthroplasties using Artek (Centerpulse, Winterthur, Switzerland) or Inter-Op (Sulzermedica, acquired by Zimmer, Warsaw, IN) acetabular components were excluded from the analysis, as they had been withdrawn from the market soon after introduction due to a high revision rate.

Statistics

Comparisons between age, gender and indications for the index procedure were made between MOM and MOP patients using Pearson's chi-square test. Critical proportion testing was used for the statistical differences in the reason and type of revision in patients with MOM versus MOP prostheses (14). Cox proportional-hazards modeling were performed to determine the effect of bearing type, head size and preoperative diagnosis on the hazard of revision and infection in particular. Cumulative curves were used to describe the rate of revision in general and infection individually. Data was consequently corrected for age and gender if

applicable. For all statistical tests, $p < 0.05$ was considered to be significant. The statistical package SPSS version 15.0 (SPSS Inc., Chicago, IL) was used.

Results

100,906 hip arthroplasties, of which 17,510 MOM and 83,396 MOP implants, were recorded to have been performed throughout Australia during the study period. Review of the registry data took place at a mean of 3.4 years after the primary procedure. Forty-two different brands of femoral prostheses were recorded (Table I) and eleven individual acetabular components were used.

Table I. Combinations with both MOM and MOP attributes and at least 10 procedures in each attribute group.

Femoral prosthesis type	Acetabular component type
ABGII [1], Accolade [1], Adapter [7], Alloclassic [2], Anthology [3], Apex [4], C-Stem [5], CBC Stem [6], CBH Stem [6], CLS [2], CPCS [3], CPT [7], Cone Stem [2], Corail [8], Edinburgh [7], Elite Plus [8], Emperion [3], Epoch [2], Exeter V40 [1], Freeman [5], Hayes Consensus [12], Integral [9], M/L Taper [2], MS 30 [2], Mallory-Head [9], Margron [13], Mayo [2], Natural Hip [7], Platform [7], Profemur Z [1], Revitan [2], S-Rom [8], SL-Plus [3], Spectron EF [3], Stability [7], Summit [8], Synergy [3], Taper Fit [10], Taperloc [9], Trabecular Metal [2], VerSys [2], Wagner [2]	Allofit [2], Bionik [14], CBF Cup [6], Delta [7], Fitmore [2], Lineage [11], Mallory-Head [9], Morscher [2], Pinnacle [8], Reflection [3], S-Rom [8]

[1] Stryker, Kalamazoo, MI; [2] Zimmer, Warsaw, IN; [3] Smith & Nephew, London, UK; [4] Omnilife Science, East Taunton, MA; [5] Finsbury Orthopaedics, Surrey, UK; [6] Synthes, West Chester, PA; [7] not known to authors; [8] DePuy Orthopaedics, Warsaw, IN; [9] Biomet, Warsaw, IN; [10] Corin, Cirencester, UK; [11] Wright Medical, Arlington, TN; [12] Consensus Orthopedics, El Dorado Hills, CA; [13] Portland Orthopaedics, St. Clair, MI; [14] Orthodynamics, Lübeck, Germany.

Fifty seven percent of the patients were female. Thirty-seven percent of the patients was aged above 75. The diagnosis was primary osteoarthritis for 88%; rheumatoid arthritis for 1% and other, not further specified, for 11%. Patients with a MOM implant were significantly younger and comprised of significantly more males (odds ratio 1.7) compared to MOP patients (Table II). The diagnosis of osteoarthritis for the initial procedure was divided equally in both bearing groups. In the MOM group the odds ratio of receiving an implant with a head size larger than 32 mm was 24.7 compared to the MOP group.

2720 revision procedures had been recorded (2.7% of total). Fifty-four percent of the patients who had a revision were male (not displayed in Table II). Forty percent of these patients were older than 75. Dislocation of the prosthesis was cited as the predominant reason for revision (910 hips). Loosening occurred in

719 hips, 500 patients had undergone revision surgery due to infection, 380 because of fractures, and 37 implants were revised due to pain. Differences were seen for revision diagnosis between MOM and MOP patients (Table III). As for MOM patients, loosening was noted as the most frequent reason for revision in contrast to dislocation of the prosthesis in MOP patients. Subsequent revision procedures involved predominantly replacement of the femoral component only in 750 patients (Table IV). The acetabular component was replaced in 673 patients and the entire prosthesis in 350 patients.

Table II. Odds ratios for demographic and clinical data of patients who had a MOM implant or a revision for infection or non-infection

	All Patients	Patients who had a MOM implant		Odds ratio	Patients who had a MOP implant		Patients who had a revision due to infection		Odds Ratio	Patients who had a revision not due to infection		Odds Ratio	Patients who did not have a revision	
Number of hips	**100906**	**17510**			**83396**		**500**			**2220**			**98186**	
		n	%		n	%	n	%		n	%		n	%
Age:														
<55	9212	3821	22	8.2*	5391	6	56	11	1.5**	232	10	1.2**	8924	9
55-64	19131	5564	32	4.8*	13567	16	105	21	1.4**	487	22	1.2**	18539	19
65-74	34977	5165	29	2.0*	29812	36	189	38	1.4**	730	33	1.0	34058	35
≥75 (ref)	37586	2960	17	1.0	34626	42	150	30	1.0	771	35	1.0	36665	37
Male	43830	9521	54	1.7*	34309	41	261	52	1.4**	974	44	1.0	42595	43
Female (ref)	57076	7989	46	1.0	49087	59	239	48	1.0	1246	56	1.0	55591	57
Indication:														
Osteoarthritis	88582	15478	88	1.0	73104	88	418	84	0.7**	1834	83	0.7**	86330	88
Other (ref)	10922	1854	11	1.0	9068	11	75	15	1.0	339	15	1.0	10508	11
Rheumatoid Arthritis	1402	178	1	0.7*	1224	1	7	1	0.7	47	2	1.1	1348	1
Head size:														
≤32mm (ref)	79642	4650	27	1.0	74992	90	393	79	1.0	1821	82	1.0	77428	79
>32mm	21263	12859	73	24.7*	8404	10	107	21	1.0	399	18	0.8**	20757	21

Ref indicates reference group of patients who had a MOM implant or a revision for infection or non-infection

* indicates a significant difference from patients that had a MOP implant ($p < 0.05$)

** indicates a significant difference from patients that did not had a revision ($p < 0.05$)

Cumulative curves as a function of time since the primary hip arthroplasties revealed that the overall 8-year revision rate was 4.9% (95% CI: 4.6-5.2%), 5.7% (95% CI: 4.9-6.7%) for MOM implants and 4.8% (95% CI: 4.5-5.1%) for MOP implants (Figure 1). MOP bearings reduced revision risk with 0.7 (95% CI: 0.72-0.78) revisions per 100 observed years compared to 1.1 (95% CI: 1.00-1.19) revisions in MOM bearings. Cox proportional hazards analysis corrected for age and gender revealed a hazard ratio for revision of MOM implants of 1.24 (95% CI: 1.12-1.37) compared to MOP implants ($p < 0.001$). When corrected for diagnosis, the

likelihood of revision for MOM prostheses compared to MOP in only osteoarthritis patients was 1.35 (95% CI: 1.21-1.50).

Table III. Data on the hips that had a revision hip arthroplasty grouped by bearing type

Reason for revision (%)	MOM implant	MOP implant
Loosening/lysis	34.9*	28.4
Infection	18.1	18.4
Dislocation of prosthesis	17.3*	33.1
Fracture	14.4	13.9
Metal sensitivity	5.1*	0.1
Pain	2.5	1.1
Leg length discrepancy	2.3	1.0
Incorrect sizing	1.6	0.5
Other	1.4	1.0
Malposition	1.0	0.7
Implant breakage acetabular	0.6	0.5
Implant breakage stem	0.4	0.4
Wear acetabulum	0.2	0.1
Instability	0.2	0.4
Heterotropic bone	0	0.2
Tumor	0	0.1
Number of arthroplasties	513	2207

* indicates a significant difference from patients that had a MOP implant ($p < 0.05$)

Table IV. Data on the procedures following revision hip arthroplasty grouped by bearing type.

Type of Revision (%)	MOM implant	MOP implant
Femoral only	30.4*	26.9
Acetabular only	30.2*	23.5
THR (femoral/acetabular)	14.6	12.5
Head only	9.6*	5.3
Head/insert	6.2*	21.3
Cement spacer	6.0	5.0
Minor components	1.9	1.9
Removal of prostheses	0.6	1.0
Insert only	0.2*	2.4
Neck only	0.2	0.0
Reinsertion of components	0.0	0.2
Bipolar head and femoral	0.0	0.0
Number of arthroplasties	**513**	**2207**

* indicates a significant difference from patients that had a MOP implant ($p < 0.05$)

The most significant determining factor of survival rates was found to be the head size in combination with the type of bearing: MOM implants with a head size > 32 mm had a significantly higher number of revision per 100 observed years (1.3; 95%CI: 1.17-1.45) compared to MOM implants with a head size ≤ 32 mm (0.8; 95% CI: 0.68-0.93) and MOP implants with head sizes > 32 mm (1.0; 95%CI: 0.85-1.18) and ≤ 32 mm (0.7; 95%CI: 0.70-0.77) (Figure 2).

The cumulative curves revealed that type of bearing also influenced infection rates (Figure 3). The overall cumulative 8-year revision rate was 1.2% for MOM implants and 0.8% for MOP prostheses. The hazard ratio for infection in the first three months after the initial operation of MOM versus MOP, adjusted for age and gender, is 0.41 (0.24, 0.72) (p =0.001). After three months the hazard ratio is 1.50 (1.16, 1.95) (p =0.002). The highest infection rates were found in MOM bearings with a head size > 32 mm (0.3 revisions per 100 observed years) compared to smaller prostheses (0.1 revisions per 100 observed years) (Figure 4).

Discussion

Worldwide increasing numbers of total hip revisions developed a demand for the comparison of bearing types and their influence on revision risk. To our knowledge, this is the first study to compare MOM versus MOP implants based on national registry data. The Australian National Joint Registry has the advantage of receiving patient data from over 290 hospitals resulting in large numbers of patients registered. Additionally MOM implants had already been introduced in Australia in 1999 and had been extensively used since by many orthopaedic surgeons (17).

The overall revision rate of 2720 out of 100,906 hip arthroplasties (2.7% of total) with a mean follow up of 3.4 years is difficult to compare with current literature, because there are no comparable patient groups with regard to the use of all the different MOM and MOP implants. In addition, despite the availability of well-established combinations with good results, Australian surgeons used 128 new combinations of prostheses in the year 2008 not previously used in 2007, resulting in a dataset less favorable for comparisons. On the whole, a 5-year survival rate of 97% is comparable with data derived from the New Zealand National Joint Registry (35).

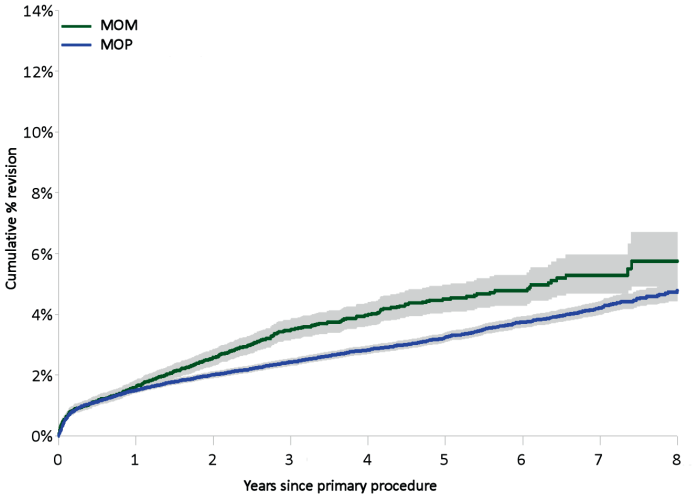

Figure 1. Yearly cumulative percent revision curve adjusted for age and gender of primary conventional THA by bearing type. The grey area represents the standard deviation.

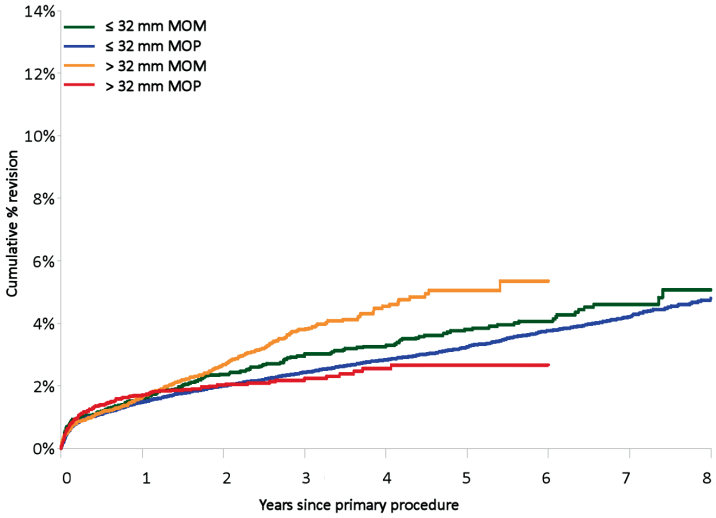

Figure 2. Yearly cumulative percent revision curve adjusted for age and gender of primary conventional THA by bearing type and head size.

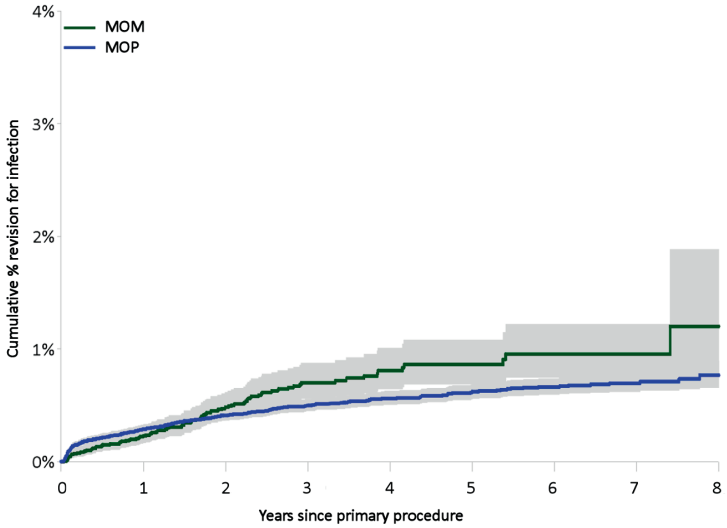

Figure 3. Yearly cumulative percent revision curve for infection adjusted for age and gender of primary conventional THA by bearing type. The grey area represents the standard deviation.

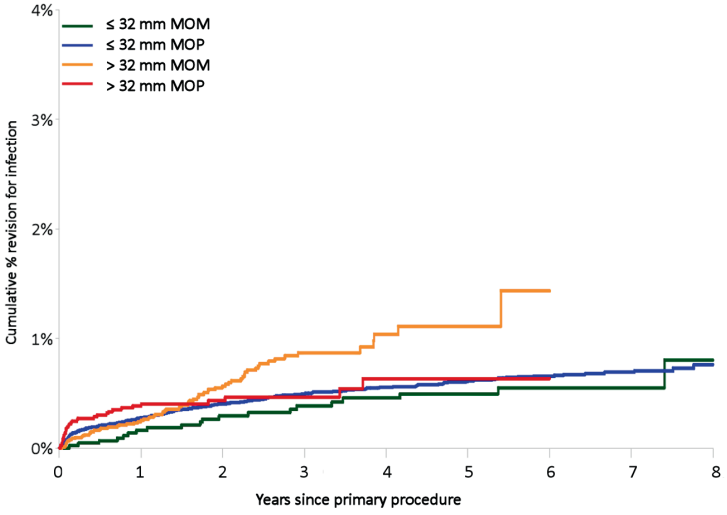

Figure 4. Yearly cumulative percent revision curve for infection adjusted for age and gender of primary conventional THA by bearing type and head size.

The most important result of this evaluation was that MOM bearings on the whole increased revision risk with a ratio of 1.6 compared to MOP bearings per 100 observed years. Due to the fact that loosening turned out to be the main reason for revision in MOM prostheses, it can be hypothesized that this prosthesis is more prone to osteolysis. When taking into account the worldwide use of MOM bearings, e.g. 35% of all prostheses in the United States in 2006 (8), this should cause some concern, especially when considering that MOM arthroplasties are generally implanted in younger patients with less co-morbidity. However, it is advised throughout the literature that caution should be applied in attributing differences in outcomes directly to the arthroplasty technology (15). The indications for hard-on-hard bearings are narrower than those for MOP and relatively young males with osteoarthritis who are currently prone to receive a MOM implant might have an increased risk for failure regardless of the implant choice (27).

High failure rates of MOM resurfacing hip implants were already reported with the use of data derived from the National Joint Registry for England and Wales (36). The 3-year revision rate was found to be 2.6% (95% CI: 2.1%-3.1%) for resurfacing implants compared to 0.9% (95% CI: 0.8%-1.1.%) in cemented prostheses. Resurfacing now constitutes only 0.5% or less of the procedures in Norway, Denmark and Sweden (19). The reason for its popularity in the United States might partially be due to initial optimistic scientific evidence, tradition and marketing policies of the manufacturers. The use of MOM implants in Australia has recently declined and a recent publication from the AOANJRR reviewing 12,093 primary resurfacing hip replacements showed a cumulative percent 8-year revision rate of 5.3 (4.6-6.2) compared to 4.0 (3.8-4.2) for conventional total hip replacement (33).

Larger head implants have been advocated in the literature for many years in order to decrease the incidence of dislocation (5;7;12) and recommendations for its usage are made in patients undergoing revision surgery (25;37). However, primary larger head sized MOM prostheses were found to have an increased revision compared to the smaller sized MOM and MOP prostheses. Randomized controlled trials may shed light if this revision rate is caused by confounders such as surgeon experience, or the individual implant designs in both groups.

Another intriguing finding from this study is the low hazard ratio for infection for MOM implants in the first three months after the initial operation (0.41) versus MOP implants. Recent research efforts towards the bacteriological influences of the MOM degradation products suggested that particulate debris reduce biofilm formation (Chapter 4). Hip simulator and clinical retrieval studies have shown that MOM hip implants commonly have an initial high wear or running-in phase, generally followed by a low wear or steady-state phase (29). It is conceivable that high running-in bacteriostatic particle-production is responsible for the low infection rate. After three months, however, the hazard ratio for infection has increased for MOM implants to 1.50 compared to MOP implants. The highest infection rates were found in MOM bearings with a head size > 32 mm. Several studies claimed that wear rates of MOM bearings turned out to be impacted in a positive way by increasing the head size (2;28). Low wear or steady-state phase will only provide a bacteriostatic effect and might not eradicate an infection focus, but may hamper the degradative function of macrophages (24).

Although we acknowledge the great value of national registries, it is questionable if the data on infection rate is reliable enough. First of all, the registry is notified of a procedure by the completion of a data form at the time of surgery. As a consequence, it is likely that there is an under reporting of infection as a revision diagnosis, as bacteriological growth from per-operatively acquired tissue samples can only be objectified after several days. Several studies also suggested a possible role for bacteria and a bacterial biofilm in 'aseptic' implant failure (21), resulting in an even higher number of false negatives. Secondly, there is also the potential for false positives, particularly with MOM prostheses in which ALVAL (aseptic lymphocyte-dominated vasculitis associated lesion) patients have the potential to be cited as revision due to an infection at the time of surgery before full microbiological and histological analysis is available.

In conclusion, the Australian National Joint Registry revealed significantly higher revision rates of MOM implants, specifically with a head size > 32 mm compared to MOP prostheses. In addition, MOM bearings with a head size > 32 mm were more prone to be revised for an infection compared to smaller and MOP implants.

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Chapter 8

General discussion

Introduction

The number of total hip arthroplasty (THA) procedures is increasing worldwide, resulting in a growing amount of patients with an infected arthroplasty (14). Infection burden for primary and revision hip prostheses is expected to increase between 2005 and 2030 from 1.4% to 6.5% and rate of revisions because of deep infection from 8.4% to 47.5% (15). Infection is a devastating complication with severe adverse consequences for the patient, i.e. re-operation, removal of the implant and increased morbidity, accompanied with high costs for society (> $500 million dollars in the U.S. (4)). Infection prevention will be of major importance for orthopaedic practice.

Until now, the two most commonly implanted hip arthroplasty bearings are metal-on-polyethylene (MOP) and metal-on-metal (MOM) articulations (3). MOP is considered to be the gold standard. Due to adverse effects of MOP wear, MOM prosthesis development was set up with the goal of manufacturing a lower wear bearing. A recent meta-analysis on MOM bearings supported their use in young and active patients (19). In addition MOM resurfacing prostheses were found comparable to MOP THA in cost-effectiveness (5). Recently, however, warnings have been issued by several orthopaedic associations advocating prudency towards MOM bearing use due to adverse soft tissue complications. We will discuss these general concerns briefly. The different studies are interpreted with specific focus on the general aim of this thesis to investigate the influence of MOM wear debris on deep infections after hip arthroplasty.

General MOM concerns

For the majority of patients, hip implants, regardless of bearing type, will improve their overall quality of life. This is also the case for MOM implants, having successfully relieved pain and improved function in the majority of all patients. Implants of all bearing types will produce wear and consequently pose risks related to the debris. However, recently, great concern has arisen among orthopaedic surgeons regarding the use of MOM bearings. Since 2007 higher revision rates were reported for individual MOM implants in the Australian Orthopaedic Association National Joint Replacement Registry (1). The ASR Total Hip System (Depuy, Warsaw, IN, USA) and Durom Acetabular Component (Zimmer, Warsaw,

IN, USA) were reported to demonstrate more than twice the risk of revision compared to other resurfacing prostheses. Attribution of doubled revision rates to surgeon's learning curves was rebutted due to the absence of such a pattern in other newly introduced prostheses. The ASR system and Durom cup are currently subject of voluntary recalls. In addition, MOM THA with head sizes over 28 mm turned out to have higher risk of revision compared to all other bearing surfaces.

In April 2010 the U.K. Medicines and Healthcare products Regulatory Agency issued a medical device alert based on data of the U.K. National Joint Registry, for specific follow-up recommendations for patients implanted with MOM implants. One year later, the U.S. Food and Drug Administration (FDA) issued a public health communication about MOM THA. However, FDA is still gathering additional information, which is complicated by the fact that the American Joint replacement Registry is not yet functional. Polls held during the American Academia of Orthopaedic Surgeons Meeting 2011 already revealed a rapid reduction in the use of MOM implants.

This thesis comprises *in vitro* studies delineating biofilm inhibitory effects of both metal ions as metal particles (**Chapter 3 & 4**), while the two final *in vivo* chapters report on an Co-Cr particle induced higher infection risk (**Chapter 6 & 7**). Albeit the narrowness of *in vitro* work is exemplified when extrapolating the conclusions of these chapters to the *in vivo* situation, all *in vitro* chapters do provide valuable detail oriented insights in the several roles of wear, holding particulate Co-Cr and its corrosion products only responsible for biofilm inhibitory effects in relatively high concentrations (**Chapter 3 & 4**), which provides more credit to the hypothesis of macrophage degradation by Co-Cr particles will be responsible for increased infection risk as proposed in **Chapter 6**. The conclusions from our work is difficult to extrapolate on its turn to the human condition depending on the shortage of long-term studies with large patient groups, yielding infection rates in MOM implants unknown (16;24). Until now, a systematic review of several smaller studies (13) and a bigger retrospective clinical cohort study (7) have lacked the numbers needed for small differences in infection rates between patient groups (Figure I). In **Chapter 7** we presented a registry study towards infection rates in MOM and MOP patients. We revealed the hazard ratio for infection three months after the initial procedure, had increased for MOM implants

to 1.50 compared to MOP implants, which was found to be in line with the results derived from **Chapter 6**, suggesting an *in vivo* hampering of the immune system in individual cases. Therefore, registry data can provide a good perspective in the field of deep infection, preferably combining data of several countries in the future.

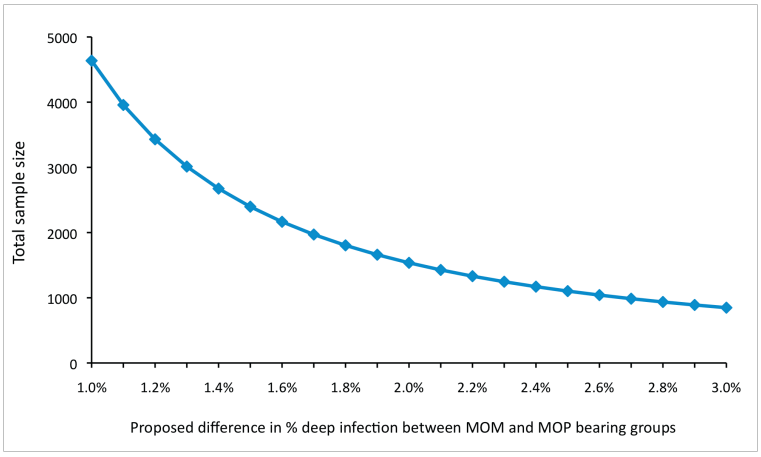

Figure 1. Numbers of patients needed to obtain a study with power = 0.8 and $p < 0.05$. *A priori* sample sizes were calculated using statistical tests for differences between two independent proportions with the statistical computer program G*Power Version 3.1.2. (University of Kiel, Kiel, Germany).

Methodological aspects of this thesis

We acknowledge two limitations towards the validity of the described studies in this thesis. First of all, particles used for *in vitro* and animal studies throughout the thesis were of larger size than clinically obtained nano-sized wear debris. As noted in **Chapter 6**, larger sized particles are not expected to influence the provoked inflammatory reactions (25), but do influence the phagocytosability, which determines whether wear debris is exposed to an acid environment inside lysosomes accelerating corrosion. However, more than 50 % of the particles used were in the phagocytosable size range smaller than 10 µm (8). Moreover, Ceridust particles have been reported to resemble UHMWPE wear debris derived from MOP implants (8;12). We chose to work with micrometer sized particles, as *in vitro* work requires large quantities of particles which are not readily available in case of nanoparticles. Moreover, microparticle size distributions could be equalized for Co-Cr and UHMWPE particles. In future experiments one may consider the use of

commercially available Cr-phosphate or Cr-oxide particles, recently found to be present in the joint tissues of MOM patients (10). However, since the final conclusion of this thesis, particularly based on our animal study, is in line with our registry analysis, we do not consider these limitations as being severe.

Future research directions

There is growing concern that metal contamination co-regulates resistance of antibiotic-resistant strains (2;23) (**Chapter 2**). We explored effects of orthopaedic biomaterials on gentamicin efficacy in **Chapter 5**, but did not investigate metal induced resistance towards antibiotics. The relationship between antibiotic and metal susceptibility has been established in general (20) for staphylococcal species against multiple metals and antibiotics (22) and in particular for Co-Cr increasing sensitivity to penicillin (17). Future research is needed to establish the clinical significance of these findings.

Alongside the use of MOP and MOM implants, already 14% of all prostheses implanted in 2006 in the U.S. contained ceramic-on-ceramic bearings (3). Wear rates of ceramic-on-ceramic bearings are low, and induce little biological reactions (11;18). Therefore, this bearing type seems promising for future use in young and active patients and necessitates investigations towards the influence of its wear on infection risk.

In THAs the concept “race for the surface” applies. This concept describes the competition between host tissue and bacteria for the colonization of the surface of implanted materials (9;21). However, this concept could also be true for the competition between individual bacterial strains. Do surfaces or wear of prosthetic components attract different bacterial species? It would be interesting to evaluate multi-strain bacterial preference for biomaterials. First of all, this would have a clinical significance in determining the infection pathway; with *S. epidermidis* being responsible for a low-grade chronic infection and *S. aureus* being responsible for acute infections (6). Secondly, non-pathogenic bacteria opposed to difficult to treat pathogens might be attracted or deterred by a certain bearing or wear type, influencing the chances of pathologic colonization of the implant.

In summary, this thesis contains an analysis of the infection risk associated with wear debris from MOM hip arthroplasties. We conclude from our *in vitro*

studies that Co-Cr ions and particles impact biofilm formation and that substrata influence the susceptibility to gentamicin. *In vivo*, animal studies in mice demonstrate a higher infection risk in the presence of Co-Cr particles compared with UHMWPE particles, possibly because the macrophage degradative function was hampered by the Co-Cr particles. Whether or not this conclusion may be extrapolated to the human situation remains to be seen, but this conclusion concurs with our comparison of the cumulative 8-year revision rate for infection MOM implants (1.2%) and for MOP prostheses (0.8%). It is an important finding that wear influences infection risk, which stimulates future research toward the influence of wear of newer bearing types on infection, possibly altering future prosthesis development.

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Summary

The main aim of this thesis was to investigate the influence of metal-on-metal (MOM) wear debris on deep infections after hip arthroplasty. The first chapter addressed the rationale behind this aim (**Chapter 1**).

A literature review (**Chapter 2**) revealed that wear products influenced the risk of infection by hampering the immune system, inhibiting or accelerating bacterial growth and by a possible antibiotic resistance and heavy metal co-selection mechanism. Whether or not the combined effects of MOM wear products make MOM bearings less or more prone to infection requires more investigation, as aimed for in this thesis.

The consequences of corrosion products of cobalt-chromium (Co-Cr) alloys are, for the most part, unclear, and the influence of Co-Cr ions on biofilm formation has never been studied. Therefore, the aim of the first *in vitro* study (**Chapter 3**) was to evaluate how Co-Cr ions affected bacterial growth, biofilm formation, and architecture. A collection of clinically isolated and commercially available bacterial strains was exposed to Co-Cr concentrations as found in serum and higher, as found in adjacent tissue. Planktonic growth of bacteria was inhibited by concentrations of 200,000/93,000 µg/L Co-Cr. Co-Cr concentrations up to 20/9.3 µg/L as reported to occur in serum, revealed no consistent influence on biofilm formation, but higher concentrations of 200,000/93,000 µg/L significantly reduced *Staphylococcus aureus* and CNS biofilm formation. As indicated by confocal laser scanning microscopy, no dead bacteria were encountered in the biofilms, and the Co-Cr ion concentrations used must be classified as growth-inhibiting and not bactericidal. The results suggested that Co-Cr ions may yield MOM prostheses less prone to biofilm formation and subsequent infection.

Next (**Chapter 4**), the influence of Co-Cr particles on biofilm formation was examined. Viable volumes of staphylococcal biofilms were determined on polystyrene in the absence and presence of Co-Cr particles and Co-Cr ions. Three clinically derived and two commercially available staphylococcal strains were grown in the presence of 200,000 µg/L Co-Cr particles or 1000/500 µg/L Co-Cr ions derived from Co-Cr salts or from particle supernatant, under static and dynamic growth conditions. A dynamic model simulated the conditions that apply for biofilm formation in the human body, as synovial fluid in mobile patients with hip prostheses is in constant motion with accompanying shear rates. Images of 24 h old

biofilms were made with confocal laser scanning microscopy and analyzed with the mathematical computer program COMSTAT, yielding the biovolume of a biofilm. X-ray photoelectron spectroscopy was performed on the particles to study their elemental surface composition. Characterization of the outer surface of the particles revealed a Co-Cr oxide layer enriched by Mo relative to the bulk concentration. Most isolates showed a tendency of reduced biofilm growth in the presence of Co-Cr particles compared to growth during exposure to metal ions, but this was only significant in one strain under the dynamic growth condition (*S. aureus* 7388). MOM wear particles were found to possess antibacterial characteristics under dynamic growth conditions.

In **Chapter 5**, metabolic activity of three *S. epidermidis* and two *S. aureus* strains in biofilms grown in tryptone soya broth on titanium-alloy (Ti), Co-Cr and ultra-high molecular weight polyethylene (UHMWPE) discs was compared and related to the efficacy of gentamicin against these biofilms. Six hours old staphylococcal biofilms were grown on Ti, Co-Cr and UHMWPE discs, after which growth was continued for another 18 h in the absence or presence of gentamicin (6 mg/mL). Biofilms were evaluated in terms of the number of adhering bacteria, their live/dead ratio and metabolic activity on the different biomaterials. The number of bacteria in the 6 h biofilms was similar on all biomaterials, with minor strain dependent differences. Metabolic activity per bacterium in 6 h old biofilms prior to growth was significantly lower in 3 out of the 5 strains in biofilms on hydrophobic UHMWPE as compared with more hydrophilic Ti and Co-Cr. The reduction of viable organisms after 18 h subsequent growth in the presence of gentamicin compared to biofilms grown in absence of gentamicin was lowest on UHMWPE, but no statistically significant correlation was found between lower metabolic activity at the onset of growth. Orthopaedic biomaterials were revealed to influence metabolic activity of staphylococcal biofilms, but metabolic activity did not correlate with staphylococcal killing by gentamicin in 24 h old biofilms.

The *in vivo* influence of Co-Cr and UHMWPE particles on the risk of infection was compared by injecting particles with or without bioluminescent *S. aureus* Xen36 in air pouches, prepared in subcutaneous tissue of immuno-competent BALB/c mice (**Chapter 6**). Bioluminescence was monitored longitudinally up to 21 days and corrected for absorption and reflection by the

particles. After termination, air pouch fluid and air pouch membranes were cultured and histologically analyzed. Bioluminescence was initially lower in mice exposed to UHMWPE particles with staphylococci than in mice injected with staphylococci only. For mice exposed to Co-Cr particles with staphylococci, bioluminescence was observed to be higher in two out of six animals compared to the presence of staphylococci alone. Accordingly, a high number of macrophages was found in the air pouch of the mouse showing the most prolonged, elevated bioluminescence. In the majority of mice, infection risk in the absence or presence of Co-Cr and UHMWPE particles appeared similar, assuming that the longevity of an elevated bioluminescence is indicative of a higher infection risk. However, the presence of Co-Cr particles yielded a higher infection in two out of six mice, possibly because the macrophage degradative function was hampered by the presence of Co-Cr particles.

Lastly, 2720 revisions from the Australian national joint replacement registry were reviewed at a mean of 3.4 years after the primary procedure, comparing the infection data on metal-on-metal (MOM) versus metal-on-polyethylene (MOP) prostheses (**Chapter 7**). Comparisons were made between age, gender, indications for the index procedure and head size of the prosthesis between patients with MOM and MOP implants using Pearson's chi-square test. Cox proportional-hazard models were performed to determine predictors of revision for infection. Cumulative curves were used to describe the infection- and non-infection revision risk. In total, 100,906 hip arthroplasties, of which 17,510 MOM and 83,396 MOP implants, were performed during our study period. Of these, 2720 prostheses were revised (2.7% of total). The overall cumulative 8-year revision rate was 5%, 6% for MOM implants and 5% for MOP prostheses. The cumulative 8-year revision rate for infection was 1.2% for MOM implants and 0.8% for MOP prostheses. MOM bearings with a head size > 32 mm had a hazard ratio for revision of 1.24 compared to MOP prostheses with a head size ≤ 32 mm. In addition, MOM bearings with a head size > 32 mm were found to be revised more often for infection (0.3 revisions per 100 observed years) compared to prostheses with a smaller head size (0.1 revisions per 100 observed years). In conclusion, the Australian National Joint Registry revealed significantly higher revision rates of MOM-implants, specifically with a head size > 32 mm compared to MOP prostheses.

The results from the studies as delineated above were interpreted in view of the general influence of MOM wear debris on deep infections (**Chapter 8**). Overall, Co-Cr particles may hamper macrophage functioning yielding an increased risk of prolonged bacterial presence post-surgery as compared with UHMWPE particles. Whether or not this conclusion may be extrapolated to the human situation remains to be seen.

Dutch summary

Het belangrijkste doel van dit proefschrift was om de invloed te bepalen van slijtagepartikels van metaal-op-metaal-heupprotheses (MOM-heupprotheses) op diepe infecties. Het eerste hoofdstuk bespreekt de klinische relevantie van dit doel (**Hoofdstuk 1**).

Uit literatuuronderzoek (**Hoofdstuk 2**) blijkt dat MOM-slijtageproducten het immuunsysteem belemmeren, bacteriegroei remmen of juist stimuleren en mogelijk verantwoordelijk zijn voor de resistentie tegen antibiotica en zware metalen. Of de gecombineerde effecten van MOM-slijtageproducten de MOM-heupprotheses meer of minder vatbaar maken voor infecties, vereist meer onderzoek en behelst het doel van dit proefschrift.

Allereerst is het onbekend wat de gevolgen zijn van de corrosie die plaats heeft bij cobalt-chromium-legeringen (Co-Cr) op bacteriële groei. Daarom was het doel van het eerste *in vitro* onderzoek (**Hoofdstuk 3**) om de effecten van de corrosieproducten te evalueren. Een verzameling van klinisch geïsoleerde en commercieel beschikbare bacteriestammen werden blootgesteld aan Co-Cr-ionenconcentraties, zoals aanwezig in het serum van patiënten met een MOM-prothese en hogere concentraties zoals verwacht in het aangrenzende weefsel. Planktonische groei van bacteriën werd geremd door concentraties van 200.000/93.000 µg/L Co-Cr-ionen. Co-Cr-ionen concentraties tot 20/9,3 µg/L, zoals aanwezig in het serum, bleken geen consistente invloed te hebben op de biofilmvorming, maar hogere concentraties van 200.000/93.000 µg/L verminderden *Staphylococcus aureus* en *Staphylococcus epidermidis* biofilmvorming aanzienlijk. Confocaal laser scanning microscopie liet weinig dode bacteriën zien in de biofilms. De gebruikte concentraties Co-Cr-ionen moeten daarom worden aangemerkt als groei-remmend en niet bacterie-dodend. De resultaten suggereren dat Co-Cr-ionen de MOM-prothesen minder gevoelig zouden kunnen maken voor biofilmvorming en een daaropvolgende infectie.

Vervolgens (**Hoofdstuk 4**) is de invloed van Co-Cr-partikels op biofilm vorming onderzocht. Het biovolume van biofilms van staphylokokken werd bepaald op polystyreen in de aan- en afwezigheid van Co-Cr-partikels en Co-Cr-ionen. Drie klinische en 2 commercieel verkrijgbare stammen werden gekweekt met 2 mg/mL Co-Cr-partikels, of met concentraties van 1000/500 µg/L Co-Cr-ionen afkomstig van Co-Cr-zouten of van het partikel-supernatant, onder statische en dynamische

groeicondities. Een dynamisch model simuleerde de condities die gelden voor een biofilm in het menselijk lichaam, zoals gewrichtsvocht dat in mobiele patiënten met heupprothesen constant in beweging is met bijbehorende flow-krachten. Beelden van 24 uur oude biofilms werden gemaakt met confocaal laser scanning microscopie en geanalyseerd met het wiskundige computerprogramma COMSTAT. Röntgen foto-elektron spectroscopie (XPS) werd uitgevoerd om de elementaire samenstelling van het partikeloppervlak te bestuderen. Karakterisering van de partikels liet een met molybdeen verrijkte oxidelaag zien ten opzichte van de bulkconcentratie. Bij de meeste bacteriestammen was er een tendens van verminderde biofilmvorming in de aanwezigheid van Co-Cr-partikels ten opzichte van de groei tijdens de blootstelling aan Co-Cr-ionen, maar dit was alleen significant in één stam bij dynamische groeicondities (*S. aureus* 7388). Samenvattend, bleken Co-Cr-partikels alleen antibacteriële eigenschappen te bezitten onder dynamische groeiomstandigheden.

In **Hoofdstuk 5** is de metabole activiteit bepaald van 3 *S. epidermidis* en 2 *S. aureus* stammen in biofilms gegroeid in tryptone-soya-groeimedium op titanium-legering- (Ti-), Co-Cr- en ultra-hoog-moleculair-gewicht-polyethyleen-discs (UHMWPE-discs) en vergeleken met de effectiviteit van gentamicine op de biofilms. Zes uur oude biofilms werden geïncubeerd op de discs van verschillend orthopedisch materiaal, waarna 18 uur additionele groei in de aan- en afwezigheid van gentamicine (6 mg/mL) volgde. De biofilms werden beoordeeld op basis van het aantal bacteriën, hun *live/dead* ratio en hun metabole activiteit. Het aantal bacteriën in de 6 uur oude biofilms was vergelijkbaar groot op alle discs, met geringe stam-afhankelijke verschillen. De metabole activiteit van bacteriën op meer hydrofobe UHMWPE-discs was lager in 3 van de 5 stammen dan in bacteriën gehecht aan meer hydrofiele Ti- en Co-Cr-discs. Het aantal levensvatbare organismen na een additionele blootstelling aan gentamicine (18h) in vergelijking met biofilms gekweekt in afwezigheid van gentamicine, was het hoogst op UHMWPE-discs vergeleken met het aantal op Ti- en Co-Cr-discs. Het orthopedische materiaal van de discs bleek van invloed te zijn op de metabole activiteit van de biofilm, maar de metabole activiteit was niet gecorreleerd met de effectiviteit van gentamicine.

In **Hoofdstuk 6** zijn de *in vivo* effecten van Co-Cr- en UHMWPE-partikels op het risico van infectie vergeleken. Hiertoe werden de partikels geïnjecteerd met of zonder bioluminescente *S. aureus* Xen36 in luchtzakjes, die geprepareerd waren in het subcutane weefsel van immuno-competente BALB/c-muizen. Bioluminescentie werd gecontroleerd met een follow-up van 21 dagen en gecorrigeerd voor absorptie en reflectie van het bioluminescente signaal door de partikels. Het vocht uit de luchtzakjes en het membraan van het luchtzakje werden gekweekt en histologisch geanalyseerd. Bioluminescentie was aanvankelijk lager bij muizen blootgesteld aan stafylokokken en UHMWPE-partikels, dan in muizen geïnjecteerd met stafylokokken alleen. Voor muizen blootgesteld aan Co-Cr-partikels met stafylokokken werd een hogere bioluminescentie waargenomen in 2 van de 6 dieren ten opzichte van de aanwezigheid van stafylokokken alleen. Er werd een groot aantal macrofagen in het luchtzakje gezien van de muis met de meest langdurige, verhoogde bioluminescentie. Bij de meerderheid van de muizen blijkt hiermee het infectierisico bij de aan- of afwezigheid van Co-Cr- en UHMWPE-partikels gelijk, uitgaande van de veronderstelling dat een hogere bioluminescentie over een langere tijdsduur indicatief is voor een hoger infectierisico. Echter, de aanwezigheid van Co-Cr-partikels zorgde voor een hoger infectierisico in 2 van de 6 muizen, vermoedelijk vanwege de aantasting van macrofagen door de aanwezigheid van Co-Cr-partikels.

Ten slotte (**Hoofdstuk 7**) werden 2720 revisies van het Australische nationale gewrichtsprothese register onderzocht, met name gericht op de infectie gegevens van MOM- versus MOP-prothesen. Vergelijkingen tussen leeftijd, geslacht, indicaties voor de primaire procedure en de kopgrootte van de prothese werden gemaakt tussen patiënten met MOM- en MOP-protheses met behulp van de Pearson chi-kwadraat test. Cox proportionele hazard-modellen werden uitgevoerd om hun invloed op het infectie-risico te bepalen. Cumulatieve curves werden gebruikt om het risico op een septische en aseptische revisie te beschrijven. 100.906 heuparthroplastieken, waarvan 17.510 MOM- en 83.396 MOP-protheses werden verricht tijdens onze studie periode. Er hadden 2720 revisies plaats (2,7% van het totaal). Het totale cumulatieve 8-jaars risico op revisie bedroeg 5%, 6% voor MOM-protheses en 5% voor MOP-prothesen. Het cumulatieve 8-jaars revisie risico voor infectie was 1,2% voor MOM-protheses en 0,8% voor MOP-prothesen.

MOM-prothesen met een kopgrootte van > 32 mm bleken een hazard ratio van 1,24 voor revisie te hebben ten opzichte van MOP-prothesen met een kopgrootte ≤ 32 mm. Bovendien, MOM-protheses met een kopgrootte > 32 mm bleken ook vaker te worden gereviseerd voor een infectie (0,3 revisies per 100 waargenomen jaren) ten opzichte van prothesen met een kleinere kopgrootte (0,1 revisies per 100 waargenomen jaar). Concluderend, liet het Australische Nationaal Register significant hogere revisie risico's zien bij MOM-protheses, in het bijzonder met een kopgrootte > 32 mm, ten opzichte van MOP-prothesen.

In **Hoofdstuk 8** werden de resultaten van de studies zoals hierboven afgebakend, geïnterpreteerd in het licht van de algemene invloed van MOM-slijtagedeeltjes op diepe infecties Wij concluderen dat Co-Cr-partikels het functioneren van macrofagen kunnen belemmeren en dat dit leidt tot een verhoogd risico op een langer durende aanwezigheid van bacteriën na een operatie in vergelijking met UHMWPE-partikels. Of deze conclusie kan worden geëxtrapoleerd naar de kliniek, valt nog te bezien.

List of publications and presentations

Comparison of techniques for correction of magnification of pelvic x-rays for hip surgery planning. B. The, J.W.J. Kootstra, A.H. Hosman, N. Verdonschot, C.L.E. Gerritsma, R.L. Diercks. *J Digit Imaging. 2007 Dec.*

A New Zealand national joint registry review of 202 total ankle replacements followed for up to 6 years. A.H. Hosman, R.B. Mason, T. Hobbs, A.G. Rothwell. *Acta Orthop. 2007 Oct.*

Association between radiographic biomechanical determinants and RSA-measured wear rates in total hip arthroplasties. B. The, A.H. Hosman, J.W.J. Kootstra, V. Kralj-Iglic, G. Flivik, N. Verdonschot, R.L. Diercks. *J Biomech. 2008 Oct*.

Metal-on-metal bearings: in vitro study on the influence of cobalt and chromium ions on biofilm formation. A.H. Hosman, H.C. van der Mei, S.K. Bulstra, H.J. Busscher, D. Neut. *J Biomed Mater Res A. 2008 Mar.*

Effects of metal-on-metal wear on the host immune system and infection in hip arthroplasty. A.H. Hosman, H.C. van der Mei, S.K. Bulstra, H.J. Busscher, D. Neut. *Acta Orthop. 2010 Oct.*

The influence of Co-Cr and UHMWPE particles on infection risk - an in vivo study in mice. A.H. Hosman, S.K. Bulstra, J. Sjollema, H.C. van der Mei, H.J. Busscher, D. Neut. *Journal of Orthopaedic research 2011.*

Influence of Co-Cr particles and Co-Cr ions on the growth of staphylococcal biofilms. A.H. Hosman, H.C. van der Mei, R. Kuijer, S.K. Bulstra, H.J. Busscher, D. Neut. *Submitted*

Killing of staphylococcal biofilms on orthopaedic materials by gentamicin. A.H. Hosman, S.K. Bulstra, H.C. van der Mei, H.J. Busscher, D. Neut. *Submitted*

Total Ankle Replacement: The New Zealand Experience. Oral presentation, Foot & Ankle Society Meeting 2006, Wanaka, New Zealand

Comparison of techniques for correction of magnification of pelvic x-rays for hip surgery planning. Oral presentation, Muscoloskeletal MRI and Interventional Neuroradiology 2007, Willemstad, Netherlands Antilles

A New Zealand national joint registry review of 202 total ankle replacements followed for up to 6 years. Oral presentation, Nordic Orthopaedic Federation 2008, Amsterdam, The Netherlands

Metal-on-metal bearings in hip arthroplasties: Influences of cobalt chromium wear debris on bacterial growth. Oral presentation, European Bone & Joint Infection Society Meeting 2009, Vienna, Austria

Metal-on-metal wear and infection in total hip arthroplasty. Oral presentation, Nordic Orthopaedic Federation 2010, Århus, Denmark

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